Large Membrane Domains in Hair Bundles Specify Spatially Constricted Radixin Activation

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The plasma membrane of vertebrate hair bundles interacts intimately with the bundle cytoskeleton to support mechanotransduction and homeostasis. To determine the membrane composition of bundles, we used lipid mass spectrometry with purified chick vestibular bundles. While the bundle glycerophospholipids and acyl chains resemble those of other endomembranes, bundle ceramide and sphingomyelin nearly exclusively contain short-chain, saturated acyl chains. Confocal imaging of isolated bullfrog vestibular hair cells shows that the bundle membrane segregates spatially into at least three large structural and functional domains. One membrane domain, including the stereocilia basal tapers and $\sim 1 \mu\text{m}$ of the shaft, the location of the ankle links, is enriched in the lipid phosphatase PTPRQ (protein tyrosine phosphatase Q) and polysialylated gangliosides. The taper domain forms a sharp boundary with the shaft domain, which contains the plasma membrane Ca^{2+} -ATPase isoform 2 (PMCA2) and phosphatidylinositol 4,5-bisphosphate [$\text{PI}(4,5)\text{P}_2$]; moreover, a tip domain has elevated levels of cholesterol, PMCA2, and $\text{PI}(4,5)\text{P}_2$. Protein mass spectrometry shows that bundles from chick vestibular hair cells contain a complete set of proteins that transport, synthesize, and degrade $\text{PI}(4,5)\text{P}_2$. The membrane domains have functional significance; radixin, essential for hair-bundle stability, is activated at the taper–shaft boundary in a $\text{PI}(4,5)\text{P}_2$ -dependent manner, allowing assembly of protein complexes at that site. Membrane domains within stereocilia thus define regions within hair bundles that allow compartmentalization of Ca^{2+} extrusion and assembly of protein complexes at discrete locations.

Introduction

Hair cells, neuroepithelial cells in the inner ear that transduce auditory and vestibular stimuli to electrical currents, provide a remarkable example of correlation of structure with function. Transduction takes place in a dedicated subcellular organelle, the hair bundle, which is composed of 30–300 stereocilia arranged in a precise staircase; each stereocilium contains a paracrystal of actin filaments, sheathed by the hair cell's plasma membrane (Gillespie and Müller, 2009). Mechanical stimuli deflect the bundle and open transduction channels, which admit K^+ and Ca^{2+} from the apical extracellular fluid, endolymph, that bathes the bundle. Bundles remove Ca^{2+} using the plasma membrane Ca^{2+} -ATPase isoform 2 (PMCA2), a calcium pump that is highly concentrated in stereocilia (Lumpkin and Hudspeth, 1998; Yamoah et al., 1998; Dumont et al., 2001). Phosphatidylinositol (PI) 4,5-bisphosphate [$\text{PI}(4,5)\text{P}_2$], a known regulator of PMCA2 (Hilgemann et al., 2001), also controls transduction and adaptation by hair cells (Hirono et al., 2004).

$\text{PI}(4,5)\text{P}_2$ is localized in hair cell plasma membranes in a strikingly nonuniform pattern; it is present in stereocilia shafts and concentrated at tips, but is absent from the taper region at stereocilia bases and from the soma's apical surface (Hirono et al., 2004). Protein tyrosine phosphatase receptor type Q (PTPRQ), a phosphatidylinositol phosphatase (Oganesian et al., 2003), presents a near-perfect reciprocal localization pattern to $\text{PI}(4,5)\text{P}_2$ (Hirono et al., 2004); PTPRQ may therefore maintain low levels of $\text{PI}(4,5)\text{P}_2$ in the apical surface and basal taper region. Steady-state degradation of $\text{PI}(4,5)\text{P}_2$ at tapers by PTPRQ would be a very inefficient way to maintain $\text{PI}(4,5)\text{P}_2$ distribution in stereocilia; more likely, $\text{PI}(4,5)\text{P}_2$ is segregated into a separate membrane domain (McLaughlin et al., 2002).

In many circumstances, members of the ezrin–radixin–moesin (ERM) family depend on $\text{PI}(4,5)\text{P}_2$ for triggering a conformation that allows activating phosphorylation (Fehon et al., 2010). Radixin is required for normal hearing in mice (Kitajiri et al., 2004) and humans (Khan et al., 2007). Although radixin has been localized to the taper region in stereocilia (Pataky et al., 2004) and potentially interacts with many functionally significant proteins present in stereocilia (J.-B. Shin and P. G. Gillespie, unpublished observations), little is known about the mechanism of activation in stereocilia.

We show here that the lipid composition of the hair bundle's membrane resembles most cellular endomembranes, except that ceramide lipids are unusually rich in *N*-palmitoyl (16:0) chains. Strikingly, polysialylated gangliosides are found in a micrometer-scale membrane domain at the stereocilia basal tapers that is physically segregated from the shaft/tip $\text{PI}(4,5)\text{P}_2$ domain; this

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Figure 1. Hair-bundle structural domains and lipid composition. **A**, Lipid composition of chick utricle hair bundles. The inner light blue pie graph indicates distribution of phospholipids, sphingomyelin, and cholesterol (mol% indicated); “Other” includes ceramide, hexosylceramide, and phosphatidylglycerol. Dark blue pie graphs indicate acyl chain compositions for the indicated lipid species (for phospholipids, the sum of the two acyl chains (denoted by number of total C atoms in both fatty acids/number of total double bonds in both fatty acids). In addition to the indicated acyl chain, sphingolipids also contain a C18 sphingosine backbone. **B**, Species distributions for hair bundles and utricular epithelium. The “Mol%” in the lipid class panel is calculated as moles of indicated lipid divided by moles of all lipids analyzed; percentage sign in individual lipid panels refers to moles of indicated acyl chain species divided by total moles of that lipid class. Values are given as mean $\pm$ SEM ($n = 6$ for each).

domain is stable even when cholesterol is extracted. These membrane domains are coextensive with protein domains; PTPRQ and PMCA2 are found respectively in the ganglioside and PI(4,5) P_2 domains. Moreover, radixin, essential for hair-cell function, is poised at the taper–shaft boundary and is activated at the border of the PI(4,5) P_2 domain. These experiments show that hair bundles have two large membrane domains, at least one of which may contain additional lipid microdomains, which are likely responsible for compartmentalization of actin dynamics, protein targeting, and mechanotransduction.

Materials and Methods

Materials. Sigma-Aldrich was the source for protease type XXIV, cholera toxin B subunit (CTB; catalog #C9903), neuraminidase (catalog #N2876), DNase I, carbenicillin, BSA, FITC-phalloidin, TRITC-phalloidin, filipin (type III), methyl- β -cyclodextrin (M β C), and phenylarsine oxide (PAO; catalog #P3075). The mouse anti-cholera toxin B antibody was from AbD Serotec (catalog #9540-0108). Formaldehyde (16% stock in sealed ampules) and glutaraldehyde (8% stock in sealed ampules) were obtained from Electron Microscopy Sciences. DME/F-12 medium was from Thermo Scientific (HyClone; catalog #SH30023.01). Buffers, salts, and other solution components were of the highest quality available. The PTPRQ antibody was a gift from Guy Richardson, (University of Sussex, Brighton, UK); PMCA2a was detected using antibody F2a (Dumont et al., 2001). Radixin was detected with mouse monoclonal antibody from Abnova (catalog #H00005962-M06), and phospho-ERM was detected using #3149 from Cell Signaling Technology.

Lipid mass spectrometry. Lipid and protein mass spectrometry used embryonic day 20 (E20)–E21 chick utricles of either sex. Hair bundles were purified from utricles by the twist-off technique (Gillespie and Hudspeth, 1991; Shin et al., 2007). To obtain utricular sensory epithelia, otoconia and otolithic membranes were removed from dissected utricles; the epithelium was then peeled off the basement membrane using an eyelash.

Lipids were extracted from hair bundles and epithelial fractions using an acidic organic phase (Bligh and Dyer, 1959) in all cases except for plasmalogens, which were extracted under neutral conditions. Quantitative analyses of lipids by nanoelectrospray ionization tandem mass spectrometry (MS/MS) were performed as described previously (Brügger et

al., 2006). Lipid analysis was done in positive ion mode on a QII triple quadrupole mass spectrometer (Waters) equipped with a nano Z-spray. Cone voltage was set to 30 V. Phosphatidylcholine (PC) and sphingomyelin (SM) detection was performed by precursor ion scanning for fragment ion 184 Da at a collision energy of 32 eV. Neutral loss scanning of m/z 141, 185, 189, or 277 Da, respectively, was applied for the analyses of phosphatidylethanolamine (PE), phosphatidylserine (PS), or phosphatidylinositol, using a collision energy of 20 eV, except for phosphatidylinositol, where a collision energy of 30 eV was applied. Precursor ion scanning of m/z 364, 390 and 392 Da was used for detection of plasmalogen species, using a collision energy of 20 eV. Hexosylceramide and ceramide were detected by precursor ion scanning for fragment ion 264 Da at collision energy 35 and 30 eV, respectively. Cholesterol was analyzed as an acetate derivate as described (Liebisch et al., 2006).

Protein mass spectrometry. Purified hair bundles were analyzed by mass spectrometry as described previously (Shin et al., 2010). Label-free protein quantitation used MS2 intensities (Spinelli et al., 2012) divided by molecular mass, normalized to the sum of all intensity/molecular mass; these normalized molar intensities (i_m) are proportional to the mole fraction of each protein (J.-B. Shin and P. G. Gillespie, unpublished observations). Data analyzed here were from a SEQUEST–X! Tandem analysis (J.-B. Shin and P. G. Gillespie, unpublished observations).

Hair cell isolation and immunocytochemistry. Hair cells were isolated from saccular epithelia of bullfrogs of either sex using methods described previously (Hirono et al., 2004) in low- Ca^{2+} saline containing 112 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 100 μ M $CaCl_2$, 3 mM D-glucose, and 10 mM HEPES, pH 7.4. Briefly, sacculi were treated with 1 mM EGTA for 15 min and then 75 μ g/ml protease XXIV (Sigma) for 30 min. After a 5 min treatment with 100 μ g/ml DNase I, the cells were isolated from the epithelium using an eyelash.

For standard immunocytochemistry, cells were fixed with 4% formaldehyde in low- Ca^{2+} saline; washed; blocked in PBS with 1% normal donkey serum, 1% BSA, and 0.2% saponin; and then incubated overnight at 4°C with primary antibodies in the blocking solution. Cells were washed and then treated with secondary antibodies (7.5 μ g/ml) and 0.25 μ M FITC-phalloidin. All samples were observed with an Olympus FV1000 confocal microscope equipped with a 60 $\times$, 1.42 NA oil plan-apochromat objective.

The rabbit anti-PTPRQ antibody (affinity purified, against the C terminus) was used at 1:250; PMCA2a was detected using 10 μ g/ml F2a,

generated in rabbit. Radixin was detected with 5 $\mu\text{g}/\text{ml}$ H00005962-M06 mouse monoclonal antibody (Abnova). Phospho-ERM was detected using 0.65 $\mu\text{g}/\text{ml}$ Cell Signaling Technology #3149, generated in rabbit.

Cholera toxin B subunit labeling and neuraminidase treatment. Isolated cells were fixed with 4% formaldehyde in low- Ca^{2+} saline, washed thoroughly with low- Ca^{2+} saline, and then treated with neuraminidase for 30 min. Neuraminidase was diluted to 0.8 U/ml with 0.1 M potassium acetate, pH 4.5, and then mixed 1:1 with the low- Ca^{2+} saline bathing the hair cells, so the final concentration was ~ 0.4 U/ml, and the final pH was ~ 4.6 . After cells were washed, they were treated with 10 $\mu\text{g}/\text{ml}$ CTB in PBS for 15 min. The cells were washed and then incubated with a mouse anti-CTB antibody in PBS for 15 min. The cells were washed, postfixed with 4% formaldehyde in PBS for 15 min, washed again, and then blocked, permeabilized, and treated with secondary reagents as above.

Other methods. Labeling of $\text{PI}(4,5)\text{P}_2$ in bullfrog hair cells was performed as described previously (Hirono et al., 2004). For PAO treatment, hair cells were isolated in low- Ca^{2+} saline. After letting cells settle for 15–20 min at room temperature (RT), cells were washed with 75% DME/F-12 medium with 18.75 $\mu\text{g}/\text{ml}$ carbenicillin. Hair cells were treated with 30 μM PAO in the same medium for 1.5 h at RT (21–22°C), then washed three times with PBS and fixed. PAO was stored as a 20 mM stock solution in DMSO. Control cells were treated with 0.15% DMSO.

Filipin was made as a 5 mg/ml stock in DMSO. Hair cells were isolated as usual and then fixed with 0.75% glutaraldehyde, 2.25% formaldehyde in PBS. After washing three times with PBS, cells were stained for 2 h with 25 $\mu\text{g}/\text{ml}$ filipin and TRITC-phalloidin. Filipin was excited using a 405 nm laser. M β C was diluted from a 2 M stock in water to a final concentration of 10 mM.

Results

Lipid composition of hair-bundle membranes

To determine the lipid composition of hair bundles, purified bundles from E20–E21 chick utricles (Gillespie and Hudspeth, 1991; Shin et al., 2007) were subjected to quantitative lipid analysis using nanoelectrospray ionization tandem mass spectrometry (Fig. 1). Despite the high sensitivity of mass spectrometry analysis, only by pooling hair bundles from many dissections were we able to readily detect bundle lipids. Each analysis ($n = 6$) used bundles from 100 chicken ears (~ 1 μg protein for each preparation); given the average size of a chick utricle stereocilium (0.25×5 μm), number of stereocilia per cell (~ 60), and bundles recovered per ear (~ 8000 , or 40%), we calculated a theoretical amount of 10 pmol per ear, which is in good agreement with the experimentally determined value of ~ 8 pmol per ear. PC, cholesterol, and PE accounted for 86% of the

Figure 2. Membrane domains in bullfrog hair bundles. **A**, Little CTB labeling without neuraminidase treatment. **B**, Pretreatment with neuraminidase greatly enhances CTB labeling, particularly in basal taper region (delineated by arrows); hair-cell apical surface labeling was also enhanced. CTB antibody used for detection in **A** and **B**. **C**, $\text{PI}(4,5)\text{P}_2$ antibody labeling. In this and other figures, $\text{PI}(4,5)\text{P}_2$ is abbreviated PIP_2 . **D**, Filipin labeling. **A–D** show isolated bullfrog hair cells; panel widths are all 12.5 μm . **E**, Approximate positions of regions used for profile averaging. **F**, Profile averages for CTB ($n = 7$), actin ($n = 7$), and $\text{PI}(4,5)\text{P}_2$ ($n = 6$). Colored lines indicate means, and the gray shading shows $\pm$ SEM. Profiles of individual cells were aligned to the dip in actin staining at the taper; this point was defined to be zero on the abscissa. **G**, Profile averages for filipin-stained cells ($n = 11$). Profiles were aligned to the peak of actin staining, which is near stereocilia tips; this point was defined to be zero on the abscissa.

lipid detected; PS, SM, and PI together made up 12%. Phosphatidylglycerol, ceramide (Cer), and hexosylceramide were also detected as minor species. We detected 182 lipid species, 76 of which accounted for 96% of the total lipid species (Fig. 1).

Comparison with lipids of utricular epithelia revealed that the overall lipid class composition of hair bundles did not differ significantly from the whole organs, except for PI, which was higher in the epithelium (Fig. 1B). However, within individual lipid classes, significant differences were observed in the species distri-

Figure 3. Ganglioside domain controls. **A**, Taper domain is visible with antibody against GM1 ganglioside (after neuraminidase treatment). As seen with some cells labeled with CTB (e.g., **D**, **E**), the lower half of the kinociliary bulb was labeled. **B**, After neuraminidase treatment, the taper ganglioside domain is visible in live hair cells treated with rhodamine-phosphatidylethanolamine (Rh-PE) to label membranes and Alexa 488-CTB (488-CTB) to label gangliosides. **C**, Additional treatment of cell in **B** with anti-cholera toxin antibody does not change the 488-CTB pattern appreciably. **D**, Control hair cell labeled with CTB after neuraminidase treatment. CTB antibody used for detection. **E**, Hair cell treated with 10 mM MβC for 40 min to extract cholesterol. The taper ganglioside domain is not disrupted. As seen occasionally, the lower halves of the kinociliary bulbs of the cells in **D** and **E** were also labeled with CTB.

butions for sphingolipids and glycerophospholipids. In hair bundles, SM and Cer species nearly exclusively contained short, saturated *N*-palmitoyl (16:0) acyl chains. For example, the 16:0 species of Cer accounted for 73% of all Cer in bundles and 33% in epithelium, but only 2% in porcine brain (B. Brügger, unpublished observations). Moreover, epithelial lipids show an unusual broad distribution of sphingolipid species. For PC and PE, hair bundles were enriched in arachidonoyl-containing species (36:4, 38:4, and 40:4), while docosahexaenoic-containing species (38:6 and 40:6) of PS were elevated in bundles compared to epithelium.

Membrane domains in hair bundles

We localized lipid domains of hair bundles using isolated bullfrog hair cells; the stereocilia of these cells have a large diameter ($\sim 0.4 \mu\text{m}$), permitting unusually clear visualization of individual stereocilia, basal stereocilia tapers, and other structures. We confirmed that PI(4,5) P_2 segregates within hair bundles, as we reported previously (Hirono et al., 2004). Because bullfrog hair cells are recalcitrant to transfection, we used immunostaining to show that PI(4,5) P_2 was absent from the basal taper region but found throughout the remainder of the hair bundle (Fig. 2C).

Gangliosides, which are sialic acid-modified, ceramide-based glycosphingolipids, have often been associated with cell signaling and membrane domains (Sonnino et al., 2007) (see Fig. 4D). To probe for gangliosides in stereocilia, we used CTB, which binds to many gangliosides but particularly tightly to GM1 (Kuziemko et al., 1996). Under standard conditions, CTB binding sites were absent from hair bundles (Fig. 2A); however, pretreatment of isolated hair cells with neuraminidase, which converts polysialylated gangliosides to GM1 (Rauvala, 1979), markedly increased the ability of CTB to label bundles (Fig. 2B). Neuraminidase-dependent labeling extended from the apical surface of the hair cell and through the stereocilia taper region, terminating a micrometer or so above the tapers in the region of the ankle links; the region labeled with CTB was exactly reciprocal of the PI(4,5) P_2 domain. This pattern was observed in at least 95% of isolated hair cells, in >15 separate experiments. The lateral membrane, segregated from the apical membrane by the remnants of the tight junctions, had much lower levels of neuraminidase-dependent CTB labeling.

Both boiling the CTB and preincubation with excess GM1 ganglioside eliminated labeling in bundles. While not labeling as strongly as CTB, an antibody specific for GM1 ganglioside gave a similar pattern after neuraminidase treatment (Fig. 3A). We were unable to identify the

specific ganglioside species responsible for the taper labeling. Although antibodies against GD1a and GT1a, two polysialylated GM1 relatives, gave no hair-bundle signal, cells potentially have many polysialylated gangliosides that can be converted into GM1 (Fig. 4D). MβC, which extracts cholesterol and often disrupts ganglioside-containing lipid domains (Simons and Sampaio, 2011), did not disrupt the basal ganglioside domain (Fig. 3E).

We usually detected gangliosides with a two-step procedure, first labeling with CTB and then amplifying the signal with an anti-CTB antibody (Fra et al., 1994; Harder et al., 1998). While

Figure 4. Hair-bundle membrane proteins and PI(4,5)P₂ pathway. In all panels, proteins indicated by blue-colored short names were detected in purified hair bundles by mass spectrometry. **A**, Chick hair-bundle membrane proteins. Proteins detected by liquid chromatography (LC)-MS/MS were quantified using intensity-weighted spectral counting. The size of each slice is proportional to molar abundance. Red, Proteins enriched in bundles over epithelium more than fivefold; green, less than fivefold. Only proteins enriched 0.1-fold or greater are plotted. **B**, Peptide coverage of PI(4,5)P₂-transport and metabolizing proteins in LC-MS/MS of chick hair bundles. Peptides detected by X! Tandem analysis of the complete dataset of chick bundles were plotted against residue number. Height of plotted bars corresponds to the number of identical peptides detected; width corresponds to peptide length, plotted at the position in sequence. Gray shading indicates peptide log(e) (log of expectation) score, indicating statistical confidence in protein identification (key is shown in PITPNA panel). **C**, PI pathway in hair bundles. **D**, Ceramide, sphingolipid, and ganglioside pathways. Green indicates lipids analyzed by mass spectrometry (Fig. 1); glucosylceramide and galactosylceramide are analyzed together as hexosylceramide. Italicized enzyme short names correspond to proteins not detected in bundles. All sialyltransferase (SAT) reactions can be reversed by endogenous or exogenous neuraminidase. Polysialylated gangliosides that could be converted to GM1 ganglioside with neuraminidase treatment are indicated with gray shading. **E**, Bundle abundance (green) and bundle-to-epithelium enrichment (magenta) of PI(4,5)P₂ and ganglioside metabolic proteins.

useful for its sensitivity, the method can generate artificially large ganglioside domains due to antibody cross-linking (Harder et al., 1998). The taper-region ganglioside domain was readily detected, however, when live (or fixed) (data not shown) hair cells were labeled with CTB modified with Alexa 488, without anti-CTB antibody (Fig. 3B). Cross-linking CTB molecules with anti-CTB had little effect on the taper ganglioside domain, although some increase in punctate labeling was seen in stereocilia shafts (Fig. 3C).

Stereocilia tips also show lipid segregation. We used the anti-biotic filipin, a fluorescent cholesterol-binding molecule, to localize cholesterol within hair cells (Bornig and Geyer, 1974). As seen in a previous electron microscopy study (Jacobs and Hudspeth, 1990), filipin strongly stained stereocilia tips (Fig. 2D,G). Filipin-detected cholesterol was present in stereocilia shafts, but appeared more abundant in the soma's apical surface.

PI(4,5)P₂ pathway in hair-bundle membranes

As PI(4,5)P₂ is usually synthesized locally within cells, we used protein mass spectrometry to identify membrane proteins, as well as proteins involved in membrane trafficking and lipid synthesis in hair bundles (Fig. 4A). As with lipid mass spectrometry, we used bundles purified from E20–E21 chick utricles. Using intensity-weighted spectral counting (Shin et al., 2007; Spinelli et al., 2012), we estimated that the 4 μm^2 plasma membrane of each stereocilium contained $\sim 7,000$ transmembrane proteins, $\sim 10,000$ peripheral membrane proteins, and $\sim 1,000$ lipid transfer molecules.

Proteins associated with PI(4,5)P₂ metabolism were readily detected in hair bundles (Fig. 4B,C,E). Lipid transfer proteins included ~ 120 molecules per stereocilium of phosphatidylinositol transfer protein α (PITPNA), which is thought to shuttle PI within cells. We also detected ~ 10 molecules per stereocilium each of type III α phosphatidylinositol 4-kinase (PIK4CA) and type II β phosphatidylinositol-5-phosphate 4-kinase (PIP5K2B), kinases that sequentially transform PI to PI(4,5)P₂; type I α phosphatidylinositol-5-phosphate 4-kinase (PIP5K1A) was also detected. In yeast, the PIK4CA ortholog Stt4 is anchored to the membrane by the scaffolding protein Ypp1 and the integral membrane protein Efr3a (Baird et al., 2008); we detected the orthologs TTC7A (by SEQUEST only) and EFR3A (by both search algorithms) in bundles. Finally, stereocilia contained ~ 3000 molecules of PTPRQ, the principal PI(4,5)P₂ phosphatase in hair bundles. The relative abundances of these proteins was also reflected by the spectral count tally for each in the complete dataset (Fig. 4B). Although antibodies against PIK4CA and PIP5K2B were insufficiently sensitive to detect these proteins by immunocytochemistry, we readily detected PITPNA, present in a punctate pattern throughout stereocilia (data not shown), and PTPRQ (Fig. 5). Thus, a complete pathway for transport, synthesis, and hydrolysis of PI(4,5)P₂ is present in hair bundles (Fig. 4C).

Although we did not detect any glycosphingolipid synthetic enzymes in stereocilia using mass spectrometry, these glycolipids are usually synthesized in the ER and Golgi and then transported to plasma membrane. While glycosphingolipids are usually de-

Figure 5. PMCA2 and PTPRQ segregate to distinct domains delineated by CTB labeling. **A**, PMCA2 localization in isolated cell labeled with F2a antibody. Note near absence of PMCA2 in basal taper region (arrows), which appears red in the color merge panel. No neuraminidase, CTB, or CTB antibody were used. **B**, PMCA2 absence from basal taper region is also clear in whole-mount tissue (arrows). **C**, Reciprocal PMCA2 and CTB labeling. **D**, PTPRQ and CTB labeling overlap. CTB antibody was used for detection in **C** and **D**. Panel widths: **A**, **C**, **D**, 12.5 μm ; **B**, 65 μm .

graded in lysosomes, we detected several enzymes of the pathway for metabolizing polysialylated gangliosides, including GLB1 (β -galactosidase), HEXA (β -hexosaminidase α), and NAGA (N -acetylgalactosaminidase) (Fig. 4*D,E*).

Membrane proteins respect the glycosphingolipid domain

The stereocilia transmembrane proteins PMCA2 and PTPRQ localized, respectively, to the $\text{PI}(4,5)\text{P}_2$ and glycosphingolipid domains. In isolated bullfrog hair cells, PMCA2 labeling extended through the upper part of stereocilia shafts, but was reduced substantially in the glycosphingolipid zone, the bottom 2 μm of the stereocilia (Fig. 5*A,C*). This PMCA2 localization was not an artifact of cell isolation, as cells in whole-mount bullfrog sacculus tissue, folded to allow high-resolution imaging, displayed similar localization (Fig. 5*B*). When cells were colabeled with the PMCA2 antibody and CTB, PMCA2 and gangliosides did not overlap significantly (Fig. 5*C*).

While PMCA2 was always excluded from the glycosphingolipid domain, the pattern of labeling seen in the upper domain varied remarkably. Stereocilia tip labeling was usually stronger than that of shafts; labeling often diminished $\sim 1 \mu\text{m}$ below stereocilia tips and then increased near the taper region. This pattern

was seen with monoclonal and polyclonal antibodies against PMCA2, and was not seen with antibody against NHE9, another stereocilia membrane protein (Hill et al., 2006). Remarkably, the PMCA2 labeling pattern appeared continuous between adjacent stereocilia, as if localization was coordinated across the gap.

As reported previously (Hirono et al., 2004), PTPRQ was located at the base of the stereocilia; PTPRQ and CTB labeling overlapped extensively (Fig. 5*D*), although CTB punctae seen in upper parts of stereocilia shafts apparently contained little or no PTPRQ.

$\text{PI}(4,5)\text{P}_2$ at the taper–shaft boundary activates radixin

The membrane-to-actin cross-linker radixin, a member of the ERM family, is concentrated at basal tapers (Pataky et al., 2004), although not as exclusively as is PTPRQ (Fig. 6*A*). Mass spectrometry indicates that the ~ 6000 molecules of radixin per stereocilium account for $>97\%$ of total bundle ERM proteins (J.-B. Shin and P. G. Gillespie, unpublished observations). As shown previously (Pataky et al., 2004), starting at the base of a hair bundle, radixin rose in concentration to a point $\sim 1 \mu\text{m}$ from the apical surface and then fell exponentially toward stereociliary tips (Fig. 6*D,E*).

Radixin interacts with membranes and membrane proteins only after activation, which requires sequential $\text{PI}(4,5)\text{P}_2$ binding and phosphorylation on T564 (Fehon et al., 2010). Once activated, radixin not only links membrane and cytoskeleton, but coordinates cellular activities by scaffolding signaling components (Neisch and Fehon, 2011). In hair bundles, radixin may interact with a large network of candidate partners identified by network analysis, including overlapping interaction with SLC9A3R2 (NHERF2) and Ras homology family member A (RHOA) networks (J.-B. Shin and P. G. Gillespie, unpublished observations).

To examine phosphoradixin distribution in stereocilia, we used a phosphospecific antibody selective for ERM proteins phosphorylated on the activating threonine (T564 for radixin). Remarkably, phosphorylated radixin was only detected above the basal tapers (Fig. 6*B–D*). The boundary was sharp and corresponded to the taper–shaft membrane-domain boundary. Above the boundary, phosphorylated radixin was elevated in a band about $\sim 0.5 \mu\text{m}$ wide and then declined exponentially toward stereocilia tips; taller stereocilia had more intense, more extensive labeling (Fig. 6*B–D*). Notably, this band was located near the ankle links and a concentration of myosin-VIIA (MYO7A) (Hasson et al., 1997), although the phosphoradixin band only partially overlapped with the MYO7A band (Fig. 6*D*).

To demonstrate the dependence of the phosphoradixin zone on $\text{PI}(4,5)\text{P}_2$, we depleted $\text{PI}(4,5)\text{P}_2$ using the $\text{PI}(4)\text{P}$ kinase inhibitor PAO. As reported previously (Hirono et al., 2004), PAO

effectively reduced PI(4,5)P₂ levels in hair bundles (Fig. 7*G–I*). Likewise, PAO reduced the level of phosphoradixin by almost 60% (Fig. 7*A, B, I*). Although PAO also affects enzymes other than PI(4)P kinase, our result is consistent with the hypothesis that localized phosphoradixin formation depends on PI(4,5)P₂. In addition, PAO treatment destabilized radixin, as total radixin in bundles declined by ~50%. This result suggests that a substantial fraction of radixin in bundles is phosphorylated, and when dephosphorylated, it exits bundles. PAO had no effect on the distribution or abundance of PMCA2 or PTPRQ (Fig. 7*C–F*).

Discussion

Hair-bundle lipid composition

The lipid composition of stereocilia membranes is similar to that of other cellular membranes; PC and cholesterol make up the bulk of the lipids, with PE, PS, SM, and PI each contributing 3% or more to the total. Acyl chains are mixed between the relatively short and saturated 34:1 PC and 16:0 SM chains, and longer unsaturated chains predominant in PE, PS, and PI. Although mass spectrometry cannot determine the leaflet distribution of each component, stereocilia devote substantial effort to properly maintaining phospholipid asymmetry (Shi et al., 2007; Goodyear et al., 2008). Indeed, the ATP8B1 P-type transporter, proposed to be responsible for translocating lipids from the extracellular to intracellular leaflet, is essential for hearing (Stapelbroek et al., 2009) and is readily detected by mass spectrometry in chick utricle hair bundles (J.-B. Shin and P. G. Gillespie, unpublished observations).

Several key lipids were not detected in our analysis. PI(4,5)P₂ is typically present at much lower levels than PI; moreover, isolated hair bundles likely deplete ATP rapidly, preventing synthesis of PI(4,5)P₂, and PTPRQ may exhaust remaining PI(4,5)P₂ before bundles can be isolated and degradation stopped. Thus the concentration of PI measured likely reflects the total PI plus PI(4)P plus PI(4,5)P₂ in intact bundles. Glycosphingolipids are not readily detected by mass spectrometry because of their scarcity and diversity; together, they account for only a few percent of all lipids, and >100 distinct glycosphingolipid species have been identified (Hakomori, 2004). Although lipidomics with high-resolution mass spectrometers allows direct detection of gangliosides (Sampaio et al., 2011), the total amount of lipid in bundles and levels of gangliosides are too low for detection at present.

Two membrane domains in stereocilia

We show here that stereocilia membranes are divided into at least three large domains, each containing specific lipids and proteins. Glycosphingolipids and PTPRQ are enriched in the taper domain, which extends from a micrometer or so above the stereocilia tapers to the apical surface of the hair cell. While

Figure 6. Radixin is activated at the PI(4,5)P₂–PTPRQ boundary. **A**, Radixin and PTPRQ are located in similar, but not entirely coextensive, patterns at the base of the hair bundle. **B**, Antibody for activated ERM proteins (including pRDX) only labels above the stereocilia tapers. Note pRDX punctae throughout bundle. Arrows indicate the pRDX-free taper region. **C**, pRDX labeling is shifted toward stereocilia tips from CTB labeling. Arrows indicate the pRDX-free taper region. **D**, The MYO7A band is more basal than the pRDX band. Arrows indicate the basal MYO7A band. **E–G**, Intensity profiles for RDX/pRDX ($n = 5$; **E**), RDX/PTPRQ ($n = 5$; **F**), and CTB/pRDX ($n = 5$; **G**) averaged from individual cells aligned at the apical-surface actin dip. Panel widths: **A–C**, 12.5 μm.

glycosphingolipids are prominent in so-called membrane rafts (Edidin, 2003; Simons and Sampaio, 2011), insensitivity of the taper domain to cyclodextrins suggests that cholesterol is not necessary for its stability. Although cholesterol is typically a component of rafts, gangliosides can form separate domains with sphingomyelin but without cholesterol (Ferraretto et al., 1997). Above basal tapers, PI(4,5)P₂ and PMCA2 are enriched in the shaft domain; however, PI(4,5)P₂ and PMCA2 both appear clustered within stereocilia shafts and are further concentrated at stereocilia tips along with cholesterol, suggesting that additional segregation of membrane components occurs. Distribution of PMCA2 and PTPRQ into shaft and taper domains did not depend on CTB, CTB antibody, neuraminidase, or cell dissociation.

The membrane domains reported here are unusually large. Although lipid domains have long been detected in artificial vesicles and in native cells (Klausner et al., 1980), stable lipid clustering on a micrometer scale is not typically seen in native cells (Simons and Sampaio, 2011). The extent and appearance of the stereocilia membrane domains could have been affected by our detection techniques, as the two-step detection could cluster CTB

Figure 7. Radixin activation depends on polyphosphatidylinositols. **A, B**, RDX and pRDX (detected with anti-pERM antibody) immunolabeling without (**A**) and with (**B**) 30 μ M PAO for 1 h. pRDX and RDX both decline after PAO treatment. **C–F**, PMCA2 and PTPRQ distribution and intensity are not affected by PAO treatment. **G, H**, PI(4,5)P₂ immunolabeling with (**G**) and without (**H**) 30 μ M PAO (1 h). PAO completely abolishes PI(4,5)P₂ immunoreactivity. **I**, Mean $\pm$ SEM for whole-bundle pixel intensity; regions of interest used for measurement included the entire bundle. Significance: ** $p < 0.01$; *** $p < 0.001$. Panel widths: **A–H**, 12.5 μ m.

Figure 8. Membrane domains in hair bundles. **A**, Relationship between radixin, phosphoradixin, and PI(4,5)P₂ profiles; data are from different sets of cells, aligned at the apical surface (small peak in each profile). The black curve is obtained by multiplying the square of the relative radixin concentration by the square of the relative PI(4,5)P₂ concentration and then normalizing to a peak of 1.0. The approximate stereocilia profile is indicated by diagram. **B**, Scanning electron micrograph of bullfrog hair bundle pseudo-colored to indicate structural and functional domains. Purple, Taper domain; green, shaft/tip domain; yellow, kinocilium domain. Inset, Transmission electron micrograph of taper region.

pentamers by antibody cross-linking. However, the glycosphingolipid domain was readily visible using CTB alone, with fixed or live cells, which demonstrates that glycosphingolipids were clustered before CTB treatment. If preexisting ganglioside domains were not present, CTB could not induce formation of a continuous, large-scale phase in stereocilia (Lingwood et al., 2008).

Physical basis of membrane domains

Lateral lipid segregation can occur due to structural dissimilarity of domains' lipid acyl chains. Hair-bundle gangliosides consist of an unusually high fraction of *N*-palmitoyl (16:0) species; both ceramide, the precursor for all gangliosides, and its metabolite sphingomyelin predominantly have a 16:0 *N*-acyl chain (Fig. 1). In contrast, brain ceramide lipids are predominantly composed of 18:0 or longer *N*-acyl chains (Ben-David and Futerman, 2010). Strikingly, the utricular epithelium as a whole is far more enriched than bundles in long-chain species of ceramide and sphingomyelin (Fig. 1B). Sphingolipids (e.g., sphingomyelin, ceramide, and gangliosides) readily form segregated membrane domains due to ceramide-moiety hydrogen bonding, polar head-group interaction, and acyl chain mismatch with glycerophospholipids (Masserini and Ravasi, 2001); the preponderance of *N*-palmitoyl species in bundles would enhance this latter effect (Holopainen et al., 2001). Together these physical features may promote lateral membrane segregation of gangliosides in hair bundles, presumably along with ceramide and sphingomyelin.

Extensive on the apical surface, it is curious that gangliosides do not extend fully throughout the stereocilia, as shown with CTB labeling. Some mechanism must control the balance of sphingolipids and glycerophospholipids in the apical membrane. Gangliosides are typically thought to be localized on convex surfaces, as their bulky head group and compact acyl chains gives them a wedge-like shape. Although the stereocilia external leaflet is highly concave where the taper enters the apical surface of the hair cell, this highly concave region is quite small and likely cannot be resolved by light microscopy. Localization of PTPRQ to stereocilia bases has been proposed to depend on active transport by myosin-VI (MYO6) (Sakaguchi et al., 2008), so presence of glycosphingolipids within the basal taper region could plausibly depend on PTPRQ, particularly if the structure of PTPRQ's transmembrane domain favored binding of short, saturated acyl chains. The glycosphingolipid domain remained even when PTPRQ was internalized, however, suggesting that once the domain formed, it was relatively stable.

Spatial constriction of radixin activation

A primary role for the hair-bundle membrane domains may be to allow precise spatial activation of radixin at the position where the ganglioside and PI(4,5)P₂ domains meet. While total radixin was abundant in the stereocilia taper region, we only saw phos-

phoradixin beginning at the ganglioside-PI(4,5)P₂ boundary; only there should PI(4,5)P₂ be present at high enough levels to preactivate radixin, allowing activating phosphorylation by an unknown kinase. Indeed, the phosphoradixin profile seen in bundles can be modeled accurately as the $[pRDX] = [RDX]^2 \cdot [PI(4,5)P_2]^2$ (Fig. 8A), suggesting that phosphoradixin formation depends steeply on the concentrations of radixin and PI(4,5)P₂. Moreover, the presence of ceramide in the taper domain could also promote radixin dephosphorylation, as is the case for ezrin (Canals et al., 2010). This phosphoradixin activation zone recalls the concentration of phospho-ERM proteins toward microvillar tips, despite the presence of total ERM proteins throughout a microvillus (Hanono et al., 2006).

Based on recruitment of the PDZ-domain protein SLC9A3R1 by ERM proteins in microvilli (Reczek et al., 1997), once stereocilia radixin is activated, we speculate that it recruits the paralog SLC9A3R2, which is present at a concentration close to that of radixin (J.-B. Shin and P. G. Gillespie, unpublished observations). SLC9A3R2, in turn, may bind to many important stereocilia proteins (J.-B. Shin and P. G. Gillespie, unpublished observations). In addition, as in other systems (Fehon et al., 2010), activated radixin may bind directly to other membrane proteins, serving as a actin-membrane connector. The role of ERM proteins is so important that in radixin's absence, hair cells upregulate the paralog ezrin, partially compensating for radixin's loss (Kitajiri et al., 2004).

Corralling PI(4,5)P₂ with glycosphingolipids

The bundle contains far more PTPRQ, which degrades PI(4,5)P₂, than it does the PI(4,5)P₂ synthetic enzymes PIK4CA, PIP5K2B, and PIP5K1A. While turnover numbers for these enzymes are not known, if PI(4,5)P₂ freely interacted with PTPRQ, present at a concentration >100-fold greater than the synthetic enzymes, it would be readily hydrolyzed. The glycosphingolipid domain may therefore act as a physical barrier to prevent PI(4,5)P₂ exchange between the hair bundle and apical surface; PI(4,5)P₂ might enter the domain infrequently because of structural mismatch with glycosphingolipid domain, but PTPRQ would be present to mop up those PI(4,5)P₂ molecules that did manage to penetrate the basal taper compartment.

Are there reasons for compartmentalization of PI(4,5)P₂ in stereocilia beyond phosphoradixin activation? PI(4,5)P₂ levels in the apical surface may fluctuate as exocytosis occurs, as fusion of vesicles with the plasma membrane is associated with PI(4,5)P₂ synthesis. In contrast, PI(4,5)P₂ controls transduction and adaptation (Hirono et al., 2004), as well as other critical molecules such as PMCA2. Formation of a discrete stereocilia PI(4,5)P₂ domain using the glycosphingolipid physical barrier thus allows precise activity control through PI(4,5)P₂ levels.

Gangliosides play an essential role in the inner ear; mice with a null mutation in GM3 synthase, which is essential for formation of most ganglioside species, transiently show some responses in an auditory brainstem response assay; however, all knockout mice are deaf by postnatal day 17 (Yoshikawa et al., 2009). The ganglioside defect could be in stereocilia; likewise, PTPRQ null mice show progressive hearing loss that is complete by several weeks of age (Goodyear et al., 2003). We thus suggest that the basal taper domain, consisting of glycosphingolipids and PTPRQ (Fig. 8B), is essential for hair-cell function, presumably by segregating PI(4,5)P₂, PMCA2, and other stereocilia components away from the soma's apical surface and allowing radixin activation in a spatially precise manner.

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