Spectrin βV adaptive mutations and changes in subcellular location correlate with emergence of hair cell electromotility in mammalians

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Edited by William E. Brownell, Baylor College of Medicine, Houston, TX, and accepted by Editorial Board Member Jeremy Nathans January 11, 2017 (received for review November 17, 2016)

The remarkable hearing capacities of mammals arise from various evolutionary innovations. These include the cochlear outer hair cells and their singular feature, somatic electromotility, i.e., the ability of their cylindrical cell body to shorten and elongate upon cell depolarization and hyperpolarization, respectively. To shed light on the processes underlying the emergence of electromotility, we focused on the βV giant spectrin, a major component of the outer hair cells' cortical cytoskeleton. We identified strong signatures of adaptive evolution at multiple sites along the spectrin-βV amino acid sequence in the lineage leading to mammals, together with substantial differences in the subcellular location of this protein between the frog and the mouse inner ear hair cells. In frog hair cells, spectrin βV was invariably detected near the apical junctional complex and above the cuticular plate, a dense F-actin meshwork located underneath the apical plasma membrane. In the mouse, the protein had a broad punctate cytoplasmic distribution in the vestibular hair cells, whereas it was detected in the entire lateral wall of cochlear outer hair cells and had an intermediary distribution (both cytoplasmic and cortical, but restricted to the cell apical region) in cochlear inner hair cells. Our results support a scenario where the singular organization of the outer hair cells' cortical cytoskeleton may have emerged from molecular networks initially involved in membrane trafficking, which were present near the apical junctional complex in the hair cells of mammalian ancestors and would have subsequently expanded to the entire lateral wall in outer hair cells.

unconventional spectrins | inner ear | F-actin cytoskeleton | cortical lattice | phylogenetics

The response of the mammalian auditory organ (cochlea) to acoustic stimuli in an extended frequency range (including high frequencies) has remarkable properties including very high sensitivity and exquisitely sharp tuning (1–3). These properties are the consequence of an evolutionary process that involved major morphological and functional changes. One of them is the emergence, in the cochlea, of the outer hair cells, a unique type of specialized sensory cells that display somatic electromotility, i.e., they undergo periodic length changes in response to the oscillation of their membrane potential evoked by the sound wave (they shorten upon depolarization and elongate upon hyperpolarization) (*SI Appendix*, Fig. S1 *A–C*). This process has endowed the mammalian auditory organ with a singular mechanism of spectral analysis of the acoustic stimulus through frequency-selective mechanical amplification (1–3), whereas spectral analysis in other vertebrates (fish, amphibians, reptiles, and birds) primarily relies on electrical tuning of the hair cells (4, 5).

An intriguing question is how the emergence of somatic electromotility is related with the evolution of individual proteins involved in this process. The electromotility of outer hair cells critically depends on the presence, in their lateral plasma membrane, of prestin (SLC26A5), a member of the solute carrier family, which undergoes conformational changes driven by the receptor potential of these cells (2, 6, 7). Prestin orthologs have been identified in all vertebrate and invertebrate species examined (8, 9), but distinct cell distributions and functional properties have been reported for nonmammalian and mammalian prestins (9–12), together with amino acid sequence signatures of an adaptive evolution in the mammalian lineage (8, 13). The electromotility of outer hair cells also relies on the unique trilaminate structure of their cylindrical lateral wall, which consists of the plasma membrane (containing millions of packed prestin molecules), the cortical lattice, and an innermost membranous apparatus forming the subsurface cisternae (1, 2, 7, 14). The cortical lattice mainly consists of circumferential actin filaments cross-linked by longitudinal spectrin filaments (1, 2, 7). These are large rod-like flexible components of the cytoskeleton made of two protein subunits, α and β, aligned side by side to form

Significance

The mammalian auditory organ harbors a subpopulation of sensory hair cells that alternately shorten and elongate in response to sound stimuli. This singular feature, called somatic electromotility, is associated with the presence of a flexible cortical network of F-actin and spectrin βV, a large unconventional β-spectrin. We show that adaptive mutations of spectrin-βV occurred in the mammalian lineage and were accompanied with substantial changes in the protein distribution within inner ear hair cells. This study illustrates how a comparison of the subcellular locations of a protein between nonmammalian and mammalian vertebrates in a given cell type, combined with branch-site model analysis of the protein family in silico, can shed light on a major mammalian structural/functional innovation.

Author contributions: L.F.F. and A.E.-A. designed research; M.C., S.P., F.P., L.F.F., and A.E.-A. performed research; A.B.E. and C.P. contributed new reagents/analytic tools; M.C., S.P., F.P., L.F.F., and A.E.-A. analyzed data; and J.-P.H., L.F.F., and A.E.-A. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. W.E.B. is a Guest Editor invited by the Editorial Board.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1618778114/-/DCSupplemental.

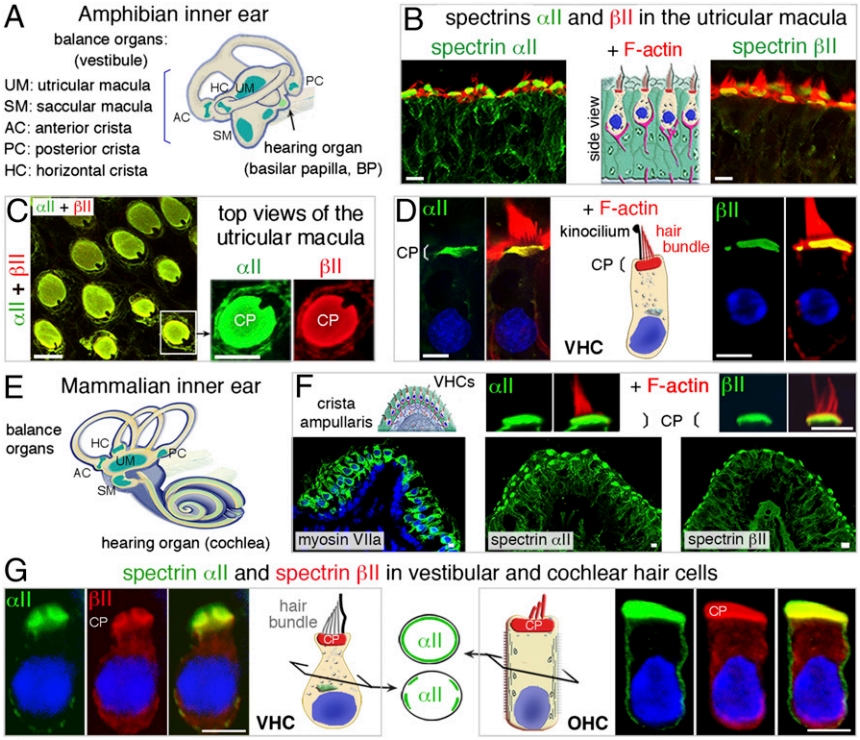

Fig. 1. Spectrins αII and βII in the frog and mouse inner ear hair cells. (*A*–*D*) Frog (*X. laevis*). (*A*) Diagram of the amphibian inner ear, showing the sensory regions of the hearing organ (light green) and of the five balance end organs (dark green). (*B* and *C*) Side (*B*) and top (*C*) views of the utricular macula. (*D*) Isolated vestibular hair cells (VHCs). In frog hair cells, spectrins αII and βII are both detected in the cell apical region, specifically, in the cuticular plate (CP) and in the submembrane region of the apical junctional complex. In *B* and *D*, F-actin is stained in red by TRITC–phalloidin. (*E*–*G*) Mouse. (*E*) Diagram of the mammalian inner ear, showing the sensory epithelia of the hearing organ (cochlea) and of the five balance end organs. (*F*) Side views of the anterior crista. The VHCs can be identified by their myosin VIIa immunostaining. As in frog hair cells, spectrins αII and βII are detected mainly in the apical region of VHCs. (*G*) In isolated VHC and cochlear outer hair cell (OHC), spectrin αII (green) and spectrin βII (red) are both detected in the CP. In the OHC, spectrin αII is also present throughout the lateral wall. By contrast, the αII immunostaining of the VHC lateral wall does not cover the entire cell circumference (see diagrams). Bars, 5 μm.

heterodimers (*SI Appendix*, Fig. S1*D*), which in turn can form oligomers through head-to-head interactions. In mammals, there are two different α isoforms (αI and αII), four conventional β isoforms (βI, βII, βIII, and βIV), and one nonconventional β isoform (βV) almost twice as long as conventional β isoforms (15, 16) (*SI Appendix*, Fig. S2). We have previously reported that the cortical lattice of outer hair cells mainly contains the αII and βV spectrin subunits (17). We now report different subcellular distributions of

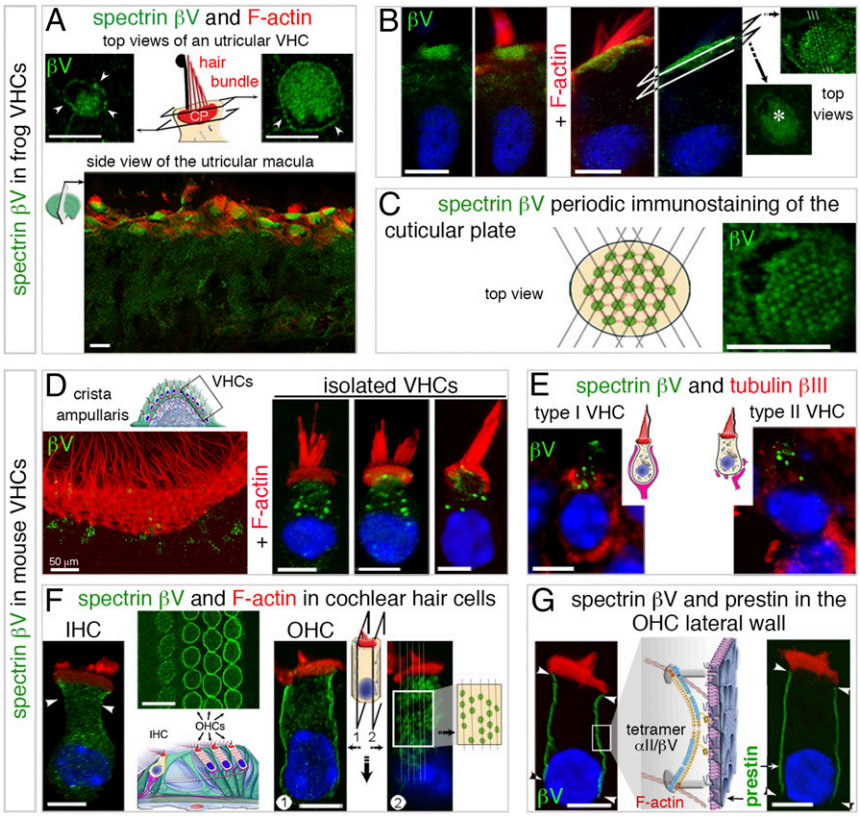

Fig. 2. Distinct subcellular distributions of spectrin βV in the frog and mouse hair cells. (*A*–*C*) Frog (*X. laevis*). (*A* and *B*) In the vestibular hair cells (VHCs) of a frog utricular macula, spectrin βV (green) is invariably detected both in the cuticular plate (CP) and as an apical immunoreactive peripheral ring (arrowheads in *A*). The top views in *B* show spectrin βV labeling just above and under (asterisk) the cuticular plate. (*C*) At the CP apical surface, the spectrin βV immunostaining forms a well-organized punctate pattern, with seemingly uniformly spaced dots resembling lattice knots. (*D*–*G*) Mouse. (*D* and *E*) In the VHCs of a mouse crista ampullaris, spectrin βV is detected as a cytoplasmic punctate immunostaining both in type I and type II VHCs, which can be differentiated by the tubulin βIII immunolabeling of their afferent nerve fibers. Spectrin βV-immunoreactive puncta extend from the apical region near the cuticular plate down to the supranuclear region of the VHCs. (*F* and *G*) In the cochlea, spectrin βV is present both in inner hair cells (IHCs) and outer hair cells (OHCs). Note that the immunostaining of the IHC lateral wall is restricted to the neck region (arrowheads), whereas the protein is detected in (and restricted to) the entire lateral wall of OHCs, where it contributes to the cortical lattice (see diagrams) (*F*). (*G*) In the OHCs, the spectrin βV immunostaining matches the distribution of the lateral plasma membrane protein prestin (arrowheads). Bars, 5 μm.

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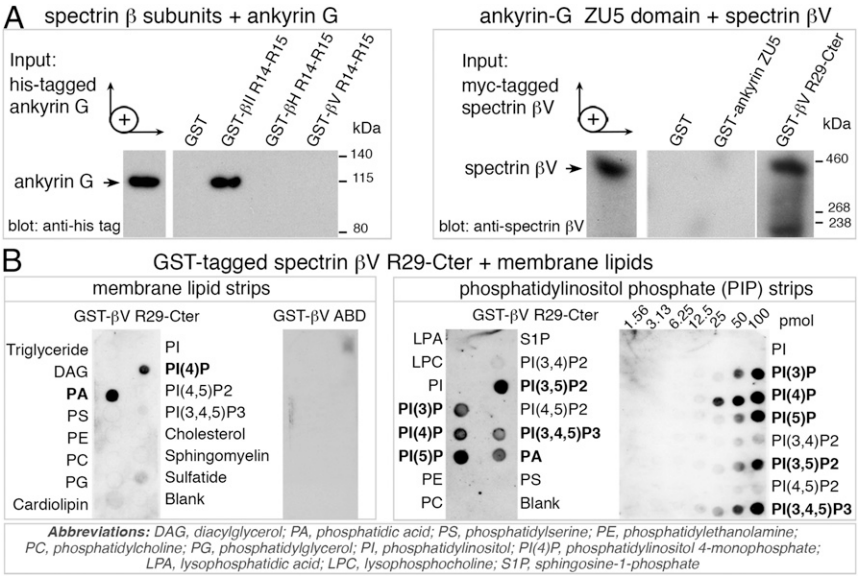

Abbreviations: *DAG, diacylglycerol; PA, phosphatidic acid; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PI(4)P, phosphatidylinositol 4-monophosphate; LPA, lysophosphatidic acid; LPC, lysophosphocholine; S1P, sphingosine-1-phosphate*

Fig. 3. Direct interaction of spectrin βV with membrane phospholipids, but not with ankyrin G. (*A*) Spectrin-ankyrin interaction. (*Left*) Purified His-tagged ankyrin G binds to a GST-tagged R14-R15 fragment of spectrin βII (GST-βII R14-R15), but not to GST-tagged R14-R15 fragments of the human spectrin βV (GST-βV R14-R15) or *Drosophila* spectrin β_H (GST-β_H R14-R15). (*Right*) In the reciprocal experiment, the GST-tagged ZU5 domain of ankyrin G (GST-ankyrin ZU5) does not bind to the myc-tagged full-length human spectrin βV. Homodimerization of spectrin βV through its C-terminal region (R29-Cter) was used as a positive control (17). (*B*) Spectrin-membrane interaction. In a membrane lipid strip assay, the R29-Cter fragment of spectrin βV, but not its actin-binding domain (βV ABD), binds to phosphatidic acid (PA) and phosphatidylinositol 4-monophosphate [PI(4)P]. In the PIP strip assay, the βV R29-Cter peptide binds to PI(3)P, PI(4)P, and PI(5)P, to PI(3,5)P2, and to PI(3,4,5)P3.

spectrin βV in amphibian and mammalian inner ear hair cells, and show how differences in protein- and lipid-binding profiles support the distinct functions of conventional and nonconventional β spectrin isoforms in these cells. In addition, a phylogenetic analysis of the spectrin–amino acid sequences in birds and mammals allowed us to identify strong signatures of adaptive evolution in spectrins αII and βV in the mammalian lineage, but not in the avian lineage. Assuming that both spectrins were present in ancestral hair cells, we suggest that special amino acid substitutions were evolutionarily selected in the mammalian lineage to serve the emerging function of sound stimulus mechanical amplification by the outer hair cells' somatic electromotility.

Results and Discussion

Differential Distribution of Spectrin βV in Amphibian and Mammalian Inner Ear Hair Cells. In the inner ear of vertebrates, the five vestibular end organs devoted to equilibration (utricle, saccule, and three semicircular canals) have remained fairly constant during evolution, whereas the auditory organ has undergone substantial changes that ultimately led to the emergence of the mammalian cochlea containing the electromotile outer hair cells (3, 18) (Fig. 1 *A* and *E*). We studied the distribution of spectrins in the frog (*Xenopus laevis*) and mouse inner ears by immunofluorescence. The typical distribution of spectrin βIII and spectrin βIV in brain neuronal cell bodies and at axonal nodes of Ranvier, respectively, was found also in the vestibular and auditory neurons (*SI Appendix*, Fig. S3 *A* and *B* and ref. 17). Spectrins αII, βII, and βV were the only spectrin subunits detected in the inner ear hair cells. In both species, spectrins αII and βII were detected mainly as a prominent, diffuse, and uniform immunostaining in the most apical region of vestibular and auditory hair cells, specifically, in the cuticular plate, a dense transverse meshwork of actin filaments located under the apical surface of these cells (Fig. 1 *A*–*G* and *SI Appendix*, Fig. S3). In contrast, the distribution of spectrin βV was different between the frog and mouse hair cells and also between the different hair cell types in the mouse. In all frog hair cells, the bulk of spectrin βV immunostaining was invariably detected underneath the apical plasma membrane, a distribution reminiscent of those of the *Drosophila*, *Caenorhabditis elegans,* and chicken nonconventional β spectrins, all of which are restricted to the apical region of epithelial cells (19–21). Detailed analysis of the immunostaining by confocal microscopy revealed a peripheral ring at the level of the apical junctional complex and also a grid-like periodic pattern overlying the cuticular plate (Fig. 2 *A* and *C*), with immunostaining becoming more and more diffuse as we went deeper into the cuticular plate (asterisk in Fig. 2*B*, *SI Appendix*, Fig. S4*A*). This may indicate a variation in the local concentration of spectrin βV, because it has previously been shown in neurons that the local concentration of β spectrin (βII in that case) is critical for its organization into a periodic skeleton (22). In the mouse vestibular hair cells (type I and type II), spectrin βV was detected as dispersed cytoplasmic immunoreactive puncta spreading from the cell apical region near the cuticular plate down to the juxtanuclear region (Figs. 2 *D* and *E* and *SI Appendix*, Fig. S4*B*). In the cochlea, the inner hair cells, which are the genuine sensory cells that make synaptic contacts with the afferent nerve fibers, displayed both a cytoplasmic punctate staining and a submembrane staining in their neck region, whereas the outer hair cells only displayed the previously reported submembrane staining of their entire lateral wall (Fig. 2 *F* and *G* and *SI Appendix*, Fig. S4*C*) (17).

Interaction of Spectrin βV with Membrane Phospholipids. Ankyrins are adapter proteins located at the plasma membrane cytoplasmic surface, where they connect diverse cell-adhesion molecules and ion transporters to the underlying cortical cytoskeleton (15, 16). All conventional spectrin β subunits interact directly, through their spectrin repeats R14-R15, with the ZU5 domain of ankyrins (15). We first showed that, as previously suggested (16, 23), nonconventional spectrin β subunits do not interact with ankyrin. Unlike the R14-R15 fragment of the mouse spectrin βII (amino acids 1694–1905) used as a positive control, the R14-R15 fragments of the human giant spectrin βV and *Drosophila* spectrin β-heavy (β_H) indeed did not bind to ankyrin G in a pull-down experiment. In the reciprocal experiment using the ZU5 domain of ankyrin G and the full-length spectrin βV, we confirmed that spectrin βV cannot bind to ZU5 (Fig. 3*A*).

Because nonconventional β spectrins do not bind to ankyrins, how do they interact with cell membranes? The previously reported direct interaction of the *Drosophila* spectrin β_H C-terminal region, including the pleckstrin homology (PH) domain, with membrane phospholipids (24) prompted us to investigate the binding of an equivalent fragment of the human spectrin βV (R29-Cter, amino acids 3317–3674), also including the PH domain, to membrane lipids in a lipid overlay assay. We found that this fragment preferentially binds to phosphatidic acid, phosphatidylinositol 3-, 4-, or 5-monophosphate, phosphatidylinositol 3,5-bisphosphate, and phosphatidylinositol 3,4,5-triphosphate

(PIP3) (Fig. 3*B*), whereas the *Drosophila* spectrin β_H preferentially interacts with PI(4,5)P2 (24). This could partially account for the targeting of these unconventional β spectrins to different cell regions or compartments. By acting as docking sites on the cytosolic side of membrane bilayers, phosphoinositides indeed play a substantial part in the targeting code (25). In epithelial cell lines such as MDCK cells, PIP3 is found in the basolateral plasma membrane and is excluded from the apical membrane, which contains large amounts of PI(4,5)P2. In addition, PIP3 is present in early endosomes, phagosomes, and autophagosomes, whereas PI(4)P functions as a trafficking regulator in the Golgi apparatus (25, 26). Therefore, βV spectrins are well suited to be recruited to different pools of cytosolic membranes, including trans-Golgi secretory vesicles.

Spectrin βV as a Cargo Adapter for Intracellular Transport in Mammalian Vestibular Hair Cells. Several studies have indicated that spectrin β_H plays a role in membrane stabilization and vesicular transport to and from the plasma membrane in the apical region of *Drosophila* epithelial cells (19, 20, 24, 27). In particular, overexpression of a C-terminal fragment of the protein, including the PH domain, in salivary glands leads to the formation of dynamin-sequestering expansions of the plasma membrane, possibly due to the down-regulation of the endocytotic machinery (28, 29). Overexpression of an equivalent fragment of the human spectrin βV (R26-Cter, amino acids 3012–3674) in HeLa cells did not have a conspicuous effect on the plasma membrane, but dramatically affected the organization of the cytoplasmic vesicular membranes. Vesicles immunoreactive for this protein fragment were detected a few hours after the cell transfection, and later coalesced into giant interconnected multivesicular compartments that invaded the entire cell body (*SI Appendix*, Fig. S5*A*).

To identify the spectrin βV-immunoreactive cytoplasmic structures in mouse vestibular hair cells, we used TRITC-conjugated wheat germ agglutinin (WGA), which labels the Golgi apparatus and Golgi-derived vesicles. We found that the WGA staining overlapped substantially with the spectrin βV immunostaining in the Golgi perinuclear region and in punctate structures extending toward the pericuticular region (Fig. 4 *A* and *B* and *SI Appendix*, Fig. S5*B*). The colocalization of spectrin βV and Golgi-derived structures was specific to spectrin βV and to vestibular hair cells, as no overlap was observed between WGA- and spectrin αII or βII stainings in vestibular hair cells (Fig. 4*C* and *SI Appendix*, Fig. S5*B*), or between WGA- and spectrin βV stainings in the cochlear outer hair cells (Fig. 4*D*). Because Rab GTPases play a key role in the transport of post Golgi-derived structures, we searched for a possible interaction between spectrin βV and Rab8, which is involved in the transport toward the plasma membrane in most epithelial cell types, including hair cells. We found that the R29-Cter fragment of spectrin βV indeed bound to Rab8 present in protein extracts of the inner ear or retina (Fig. 4*E*). Together, these results suggest that in mammalian vestibular hair cells, spectrin βV, through its interaction with Rab8, but without the assistance of spectrin αII, is involved in the directional translocation of tethered vesicular membranes to the plasma membrane. This conclusion is further supported by the previously reported interaction of spectrin βV with the dynein and kinesin complexes and with myosin VIIa, motor proteins that move cargos along microtubules and along actin filaments, respectively (30). Therefore, the involvement of nonconventional β spectrins in cargo transport seems to be evolutionarily conserved, despite differences in the subcellular location of these proteins between nonmammalian and mammalian cells. The transport function, restricted to the cell apical region in nonmammalian species, would have expanded to the entire cytoplasmic region in mammalian vestibular hair cells (and retinal photoreceptor cells), as inferred from the broad distribution of spectrin βV in these cells (see Fig. 2*B* and ref. 30).

Spectrin βV and the Emergence of Somatic Electromotility. In the cochlear outer hair cells, the reverse-transduction process transforming the electrical receptor potential into a mechanical force is powered by the prestin molecules in the lateral plasma membrane. The cortical cytoskeleton, which is ~25 nm distant from the plasma membrane, is an integral part of the force translation into the changes in cell length and stiffness that characterize somatic electromotility (1–3, 7). Possibly selected evolutionarily by virtue of its extended length, spectrin βV, with its additional 13 spectrin repeats, is by far the most abundant β subunit of the outer hair cell lateral wall, and its distribution matches that of prestin (refs. 6, 17, and Fig. 2*B*). The presence of spectrin βV in the lateral wall provides the outer hair cell with a scaffold, both robust and flexible, that directly relays the

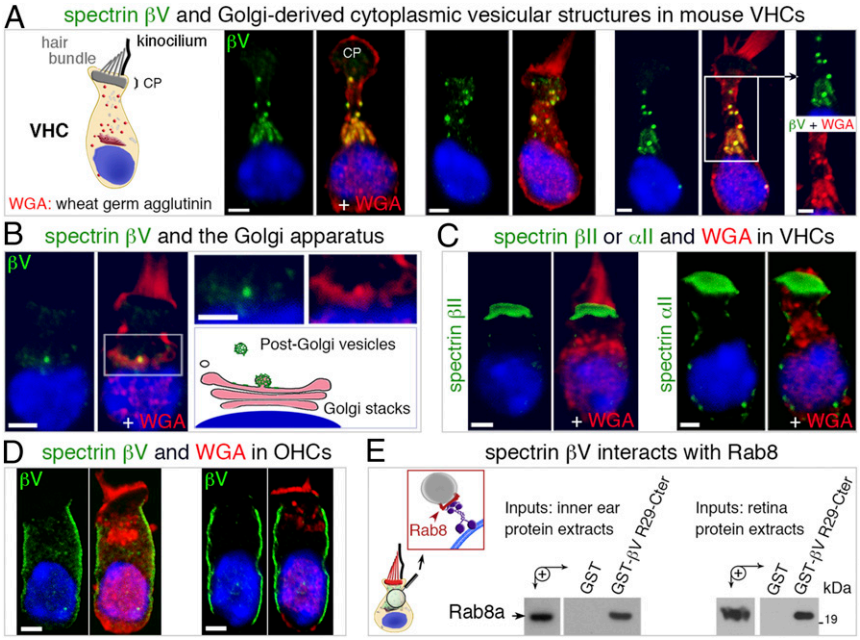

Fig. 4. Spectrin βV associates with post-Golgi structures and binds to the Rab8 GTPase. (*A*) In mouse isolated vestibular hair cells (VHCs) on P10, the staining with TRITC-tagged wheat germ agglutinin (WGA) substantially overlaps with the spectrin βV immunostaining, both in the Golgi apparatus and in scattered post-Golgi secretory vesicles. (*B*) Focus on a spectrin βV-immunoreactive and TRITC-WGA-stained spot in the Golgi apparatus. (*C*) The TRITC-WGA–stained structures (red) are not colocated with spectrin αII- and spectrin βII-immunoreactive structures (green). (*D*) Unlike in VHCs, spectrin βV in mouse cochlear outer hair cells (OHCs) does not colocalize with TRITC-WGA–stained structures. (*E*) Spectrin βV binds to Rab8, a small GTPase involved in vesicular trafficking toward the plasma membrane. A GST-tagged R29-Cter fragment of spectrin βV (GST-βV R29-Cter), but not GST alone, binds to Rab8 present in protein extracts from the mouse inner ear or retina. Bars, 2 μm.

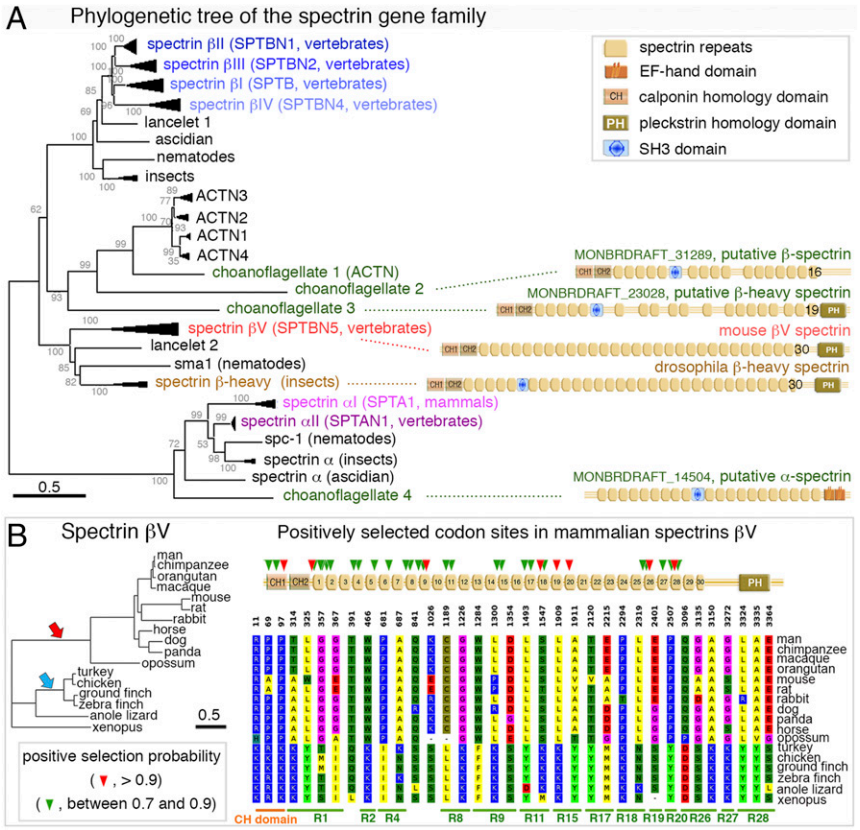

Fig. 5. Spectrin family tree and signatures of positive selection in the amino acid sequences of mammalian spectrins βV. (*A*) Neighbor-joining phylogenetic tree of the complete spectrin gene family based on a multiple full-length sequence alignment from vertebrate and invertebrate species (Jones–Taylor–Thornton amino acid substitution model). Vertebrate spectrin branches were collapsed for the sake of clarity (see detailed tree in *SI Appendix*, Fig. S7). Predicted domains of the spectrins identified in the choanoflagellate *Monosiga brevicollis* are shown on the right side of the corresponding tree branches: MONBRDRAFT_14504, _31289, and _23028 refer to the putative α, β, and β-heavy (β_H) spectrins, respectively. The nonconventional β spectrins of *Drosophila* (β-heavy) and mouse (βV) are shown for comparison. Bar, 0.5 substitution per site. (*B*) Diagram showing the positions of the amino acid residues submitted to positive selection in the mammalian lineage for spectrin βV (35 codon sites). Corresponding positions in the different vertebrate species are shown, with upper numbers indicating the positions in the human protein sequence. The protein domains are indicated below by horizontal bars. The phylogenetic tree of this spectrin is shown on the left side (the red and blue arrows indicate the mammalian and avian focal branches, respectively). Bar, 0.5 substitution per site. The detailed results of the phylogenic molecular analysis are presented in *SI Appendix*, Table S2. CH, calponin homology domain; R*n*, spectrin repeat number *n*; PH, pleckstrin homology domain.

membrane-based, voltage-dependent motor activity of prestin to the underlying cytoskeleton. In addition, the spectrin αII/βV crosslinks are expected to favor the longitudinal over circumferential mobility of lipids within plasma membrane microdomains, which may also be relevant for the cell electromotility (31).

The cochlear outer hair cells are unique to mammals, but the existence of somatic electromotility in nonmammalian species has been a matter of debate (2, 3, 13). Indeed, it has been suggested that a prestin-dependent amplification mechanism also exists in the chicken auditory short hair cells, which could be correlated with the presence of prestin in the plasma membrane of their apical circumference (12). Recent findings did support the idea that avian hair cells produce mechanical force in vivo, but indicated that the effect on the vibration of the auditory epithelium is insufficient to sharpen tuning (5). In all frog hair cells and in the mouse vestibular hair cells and cochlear inner hair cells, spectrin βV was detected near the apical junctional complex. In addition, a trilaminate structure of the lateral wall, including a lateral cistern and associated pillar and filament complexes, has been reported in the apical region of inner hair cells, which however do not display electromotility (32). Together, these results support a scenario where the trilaminate organization of the mammalian outer hair cells' lateral wall would have emerged from structures and molecular networks initially present near the apical junctional complex in the hair cells of mammalian ancestors. This putative apico-basal spreading along the hair cell lateral wall in the course of evolution may have left ontogenic traces in the present mammals, specifically, the expansion of the spectrin βV and prestin distributions from the neck region of the outer hair cells to their entire lateral wall during development, which is concomitant to the onset of electromotility (6, 17) and *SI Appendix*, Fig. S6). Likewise, the spreading of an actin- and spectrin-based cortical lattice has been reported in developing neuronal cells: the cortical skeleton of axons, a periodic structure formed by actin rings connected by flexible filaments made of βII, βIII, or βIV spectrin, is initiated next to the cell body and gradually extends to the axon terminals (22, 33, 34).

Adaptive Evolution of the Spectrin βV Amino Acid Sequence in the Mammalian Lineage. We built a phylogenetic tree of the spectrin gene family, based on multisequence alignments from vertebrate and invertebrate protein-coding sequences (Fig. 5*A* and *SI Appendix*, Fig. S7 and *SI Appendix*, Table S1). In addition, we analyzed the 13 genes encoding proteins that contain spectrin repeats in the unicellular choanoflagellate *Monosiga brevicollis*, one of the closest living relatives of metazoans, and identified three genes encoding spectrins. Prediction of protein domains allowed us to differentiate an α spectrin, a putative conventional β spectrin, and a putative β_H spectrin. The latter contains 19 predicted spectrin repeats, two N-terminal calponin homology (CH) domains, a src homology 3 (SH3) domain (also present in the β_H spectrins of invertebrate metazoans), and a C-terminal PH domain (16), whereas the putative conventional β spectrin only contains 16 predicted spectrin repeats and lacks a C-terminal PH domain (Fig. 5*A*). This result indicates that the differentiation between nonconventional and conventional β spectrins occurred in a unicellular ancestor of metazoans and preceded the appearance of ankyrins because the genome of *M. brevicollis* does not contain any ankyrin gene ortholog. The absence of a direct physical interaction between ankyrin and nonconventional β spectrins shown here (see Fig. 3*A*) further suggests that the binding to ankyrins was an evolutionary acquisition of conventional β spectrins in metazoans. Finally, a phylogenetic analysis of the modular structure of nonconventional β spectrins also allowed us to identify two substantial changes during evolution of metazoans, i.e., the addition of several spectrin repeats up to a total of 30 such repeats present in the β_H spectrins of invertebrates and the βV spectrins of vertebrates (19, 20, 27), and the removal of the SH3 domain, which is present in the two β spectrins of *M. brevicollis* and in the β_H spectrins of invertebrates, but not in the βV spectrins of vertebrates (see Fig. 5*A*).

In the course of evolution, most protein-coding DNA sequences are dominated by purifying (or negative) selection, i.e., the removal of functionally deleterious mutations. Rarely, a protein sequence will experience a burst of adaptive changes, driven by Darwinian (or positive) selection, that increase fitness. Comparative phylogenetic analyses of prestin and other SLC proteins have revealed significant adaptive changes in the amino acid sequence of prestin that occurred after the split between the avian and mammalian lineages. These changes have been related to the loss of ion transport activity of the protein in mammalians and the emergence of somatic electromotility in a subset of auditory hair cells, i.e., the outer hair cells (8, 13). We sought evidence of adaptive changes in the nucleotide sequences encoding all members of the α and β spectrin families using the modified branch-site model A, test 2 of positive selection, which allows for the selection intensity to vary among amino acid codon sites and among phylogenetic branches (*SI Appendix, Materials and Methods*). We tested the focal branches (tested branches of an evolutionary tree) in the lineage leading to mammals and the lineage leading to birds, for which multiple sequences of high quality were available. All β subunits, except βII, displayed signatures of positive selection in the mammalian lineage. The nonconventional βV subunit showed strong signatures of positive selection only in the lineage leading to mammals ($P < 1.0\ e^{-5}$). These signatures were identified at a total of 35 codon sites corresponding to amino acid positions in the first CH domain (CH1) and in several spectrin repeats (R), including R1 and R2 that are involved in protein dimerization (15). Three codon sites in the αII subunit, which forms heterodimers with βV, also displayed strong signatures of positive selection in the lineage leading to mammals ($P = 1.2\ e^{-3}$) (Fig. 5*B* and *SI Appendix*, Fig. S8 and *SI Appendix*, Table S2).

The adaptive mutations in the amino acid sequence of spectrin βV are not sufficient to account for the changes of subcellular localization of the protein that occurred in the lineage leading to mammals because we were able to show that this localization differs substantially between vestibular and cochlear hair cells in a given species (mouse). However, they presumably contributed to these changes by allowing new physical interactions with as yet unidentified binding partners that would be produced or located differentially between the different cell types, as already suggested for the phosphoinositide code (25, 26). Although prestin might also appear as a good candidate at least in the outer hair cells, previous experiments have led to the conclusion that a direct interaction between this protein and spectrin βV is unlikely (17). Whatever the precise effects of these amino acid substitutions in spectrin βV, we suggest that they were evolutionarily selected in the mammalian lineage to serve the emerging function of sound stimulus mechanical amplification by the outer hair cells' somatic electromotility, as previously suggested also for prestin and the α10 subunit of the acetylcholine receptor (8). From an evolutionary point of view, the critical role played by the cochlear outer hair cells in spectral analysis of acoustic signals and high-frequency hearing is indeed of utmost importance for the survival of most mammalian species (2, 3, 7).

Materials and Methods

A detailed description of the methods is available in *SI Appendix, Materials and Methods*. Approval for the experiments using animals was obtained from the Animal Use Committee of INSERM and Institut Pasteur. For immunofluorescence and immunoblot analyses, the reported antibodies were used as described in ref. 30. Protein–protein and protein–lipid interaction assays were carried out as described in ref. 17. In silico phylogenetic analyses on the complete spectrin family were carried out as described in ref. 8.

ACKNOWLEDGMENTS. We thank J. Boutet de Monvel for assistance in data analyses, D. Rocancourt and J. Gros for providing us with avian tissue samples, and S. Gribaldo for fruitful discussions. This work was supported by Agence Nationale de la Recherche (ANR) as part of the second "Investissements d'Avenir" program (ANR-15-RHUS-0001), Laboratoire d'Excellence (LABEX) Lifesenses [ANR-10-LABX-65], LHW-Stiftung, FAUN-Stiftung (Suchert Foundation), and grants from ANPCyT from Argentina (to L.F.F.) (PICT2012-0414; PICT2013-1642). M.C. and S.P. benefited from fellowships from Ministère de l'Education Nationale de la Recherche et de Technologie (MNERT) and Fondation Retina-France, and F.P. received a CONICET doctoral fellowship.

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