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An Isoform of GTPase Regulator DOCK4 Localizes to the Stereocilia in the Inner Ear and Binds to Harmonin (USH1C)

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The driving forces for the regulation of cell morphology are the Rho family GTPases that coordinate the assembly of the actin cytoskeleton. This dynamic feature is a result of tight coupling between the cytoskeleton and signal transduction and is facilitated by actin-binding proteins (ABPs). Mutations in the actin bundling and PDZ domain-containing protein harmonin are the causes of Usher syndrome type 1C (USH1C), a syndrome of congenital deafness and progressive blindness, as well as certain forms of non-syndromic deafness. Here, we have used the yeast two-hybrid assay to isolate molecular partners of harmonin and identified DOCK4, an unconventional guanine exchange factor for the Rho family of guanosine triphosphatases (Rho GEF GTPases), as a protein interacting with harmonin. Detailed molecular analysis revealed that a novel DOCK4 isoform (DOCK4-Ex49) is expressed in the brain, eye and inner ear tissues. We have further provided evidence that the DOCK4-Ex49 binds to nucleotide free Rac as effectively as DOCK2 and DOCK4 and it is a potent Rac activator. By immunostaining using a peptide antibody specific to DOCK4-Ex49, we showed its localization in the inner ear within the hair bundles along the stereocilia (SC). Together, our data indicate a possible Rac-DOCK4-ABP harmonin-activated signaling pathway in regulating actin cytoskeleton organization in stereocilia.

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Keywords: DOCK4; GTPase regulator; harmonin; actin bundling protein

Introduction

The actin cytoskeleton mediates a variety of physiological functions in all eukaryotic cells, including its morphology, polarity, motility and its interactions with neighboring cells.[1] These processes involve the coordinated assembly, disassembly, cross-linking, and bundling of actin filaments, and are believed to regulate the mechanical properties of the cell. Members of the Rho family of small GTPases have emerged as key regulators in controlling actin filament dynamics and network organization, through their interaction with multiple target proteins.[2,3] Studies on the inherited deafness syndromes have led to the identification of proteins essential to stereociliary function. Mutations in the triple PDZ (post-synaptic density, disc-large, zonula occludens) domain-containing protein harmonin are responsible for USH1C, an autosomal recessive disorder characterized by congenital sensorineural deafness, vestibular dysfunction and blindness due to progressive retinitis pigmentosa.[4] Defects in the *USH1C* gene also underlie the DFNB18 form of isolated deafness.[5,6] Alternatively spliced transcripts of harmonin generated from 28 exons predicts at least ten protein isoforms which can be grouped into three classes: harmonin a, b, and c.[4] The harmonin class b isoforms are expressed specifically in the inner ear, whereas other isoforms have broader tissue distribution patterns.[4,7] In the sensory cells of the inner ear,

Abbreviations used: ABP, actin-binding protein; SC, stereocilia; RT-PCR, reverse transcription polymerase chain reaction; PAB, parallel actin bundle; GEF, guanine nucleotide exchange factor; GST, glutathione *S*-transferase; GER, greater epithelial ridge.

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harmonin was reported to interact with four other known USH1 proteins, forming a scaffolding complex required for the organization of the hair bundle in the inner ear.[8,9] The domain structure of harmonin suggests that it is involved in binding different proteins, but these vary depending on the tissue and/or cellular context. Therefore, the identification of tissue-specific partners of harmonin may shed light on its role in auditory signal transduction.

We have previously identified a novel guanine nucleotide exchange factor (GEF), DOCK4, which appears to act as a nodal point or a bottleneck in a cellular signaling pathway(s) regulating tumor progression to invasion.[10] This pathway has evolved from a genetic program that regulates programmed cell death or apoptosis in *Caenorhabditis elegans*[11] and has led to the identification of novel regulators of the small GTPase ced-10 (Rac1). Specifically, ced-2 (mammalian Crk), ced-5 (mammalian DOCK180) and ced-12 (mammalian ELMO1-2), together regulate the mammalian ortholog of Rac1, ced-10.[12]

Here, we have used the yeast two-hybrid system to identify molecular partners for the scaffold protein harmonin, used as bait to screen a human brain cDNA library. One of the proteins, which we identified as an interacting protein with harmonin, was DOCK4.[10] We further confirmed the interaction of harmonin with DOCK4 in mammalian cells. Interestingly, reverse transcription polymerase chain reaction (RT-PCR) analyses revealed that there is an isoform of DOCK4, generated by alternative splicing near the C terminus. This isoform is expressed in the brain, inner ear and eye tissues and is a strong Rac activator. By immunohistochemistry analysis with isoform-specific antibody, we show that DOCK4 immunoreactivity is localized within the stereocilia. Based on these observations, we propose that a splice variant of DOCK4 binds to harmonin, highlighting the close relationship between PDZ-containing proteins and Rho GTPases in the stereocilia.

Results

Yeast two-hybrid screening for harmonin-interacting proteins

A fusion protein containing the GAL4 DNA-binding domain and harmonin (PDZ-CC1) was used as a bait in the yeast two-hybrid screening of a human brain cDNA library fused to the GAL4 transactivator domain. The presence of PDZ domains in harmonin implies that it may be involved in binding directly to the carboxyl termini of target proteins.[13] Among 45 primary yeast transformants showing positive interaction, we identified one clone of ~2000 bp, which by analysis of the Blast database revealed to be identical to the C-terminal portion of KIAA0716 (GI: 40788337, DOCK4 amino acid residues 1187–1966). *DOCK4* (GI: 29568108) is a member of the CDM (ced-5, DOCK180, Myoblast City) gene family, implicated in the regulation of Rac GTPase signaling.[10,14] The overall structure of DOCK4 is highly similar to other CDM family members, defined by its founding members, *C. elegans* ced-5, vertebrate DOCK180, and *Drosophila* Myoblast City;[11,15,16] it has an N-terminal SH3 domain, and a C-terminal proline-rich region, which appears to be unique to each family member. DOCK4 also contains the DOCK homology regions, namely DHR1 involved in binding to phosphoinositol (3,4,5) triphosphate,[17] and DHR2 or "docker" domain that has been implicated in nucleotide free GTPase binding and activation.[18] In addition to these domains, two other motifs are present in DOCK4: a poly-proline-rich C-terminal region and a putative binding site (X-T/S-X-L/F) for PDZ domains (PDZ-binding interfaces, PBIs) at the C terminus fitting the consensus for class I PBIs.[19]

Interaction between harmonin and DOCK4 in mammalian cells

We further confirmed interaction between harmonin and DOCK4 (KIAA0716) *in vitro* by transiently co-transfecting HEK293T cells with Myc-tagged harmonin full length (BC016057) and Flag-tagged pCMV-KIAA0716 constructs. We detected by Western blot analysis with anti-Myc antibody, the presence of Myc-harmonin fusion protein that was co-immunoprecipitated with Flag-tagged KIAA0716 (Figure 1(b)). Total cellular lysates from transfected cells were also included in the blot to determine levels of expression of the proteins. We subsequently showed that KIAA0716 interacts specifically with the PDZ1 domain of harmonin in mammalian cells (Figure 1(c)). This result was further confirmed through experiments using a GFP-tagged wild-type DOCK4 and pRK-Flag-harmonin-PDZ1 constructs for transfection of HEK293T cells. Immunoprecipitation with an anti-Flag antibody and subsequent Western blot analysis using an anti-GFP antibody revealed a ~240 kDa protein corresponding to GFP-DOCK4 (Figure 1(d), lane 4; top panel). The harmonin-PDZ1 protein is detected by immunoprecipitation and subsequent Western blot analysis using anti-Flag antibody (Figure 1(d), lane 4; bottom panel).

KIAA0716 is a novel isoform of DOCK4

The sequence alignment of *KIAA0716* with the 3′ end of *DOCK4* shows that it differs from wild-type *DOCK4* in that the exon 49†, 114 bp in size is not spliced into the mRNA. To determine whether the two different transcripts result from alternative mRNA splicing, we performed RT-PCR on a fetal brain cDNA library using primers located in the 3′end of *DOCK4*. We then cloned the PCR products into TOPO cloning vectors. Sequencing analysis of

† http://www.ensembl.org/Homo_sapiens/exonview

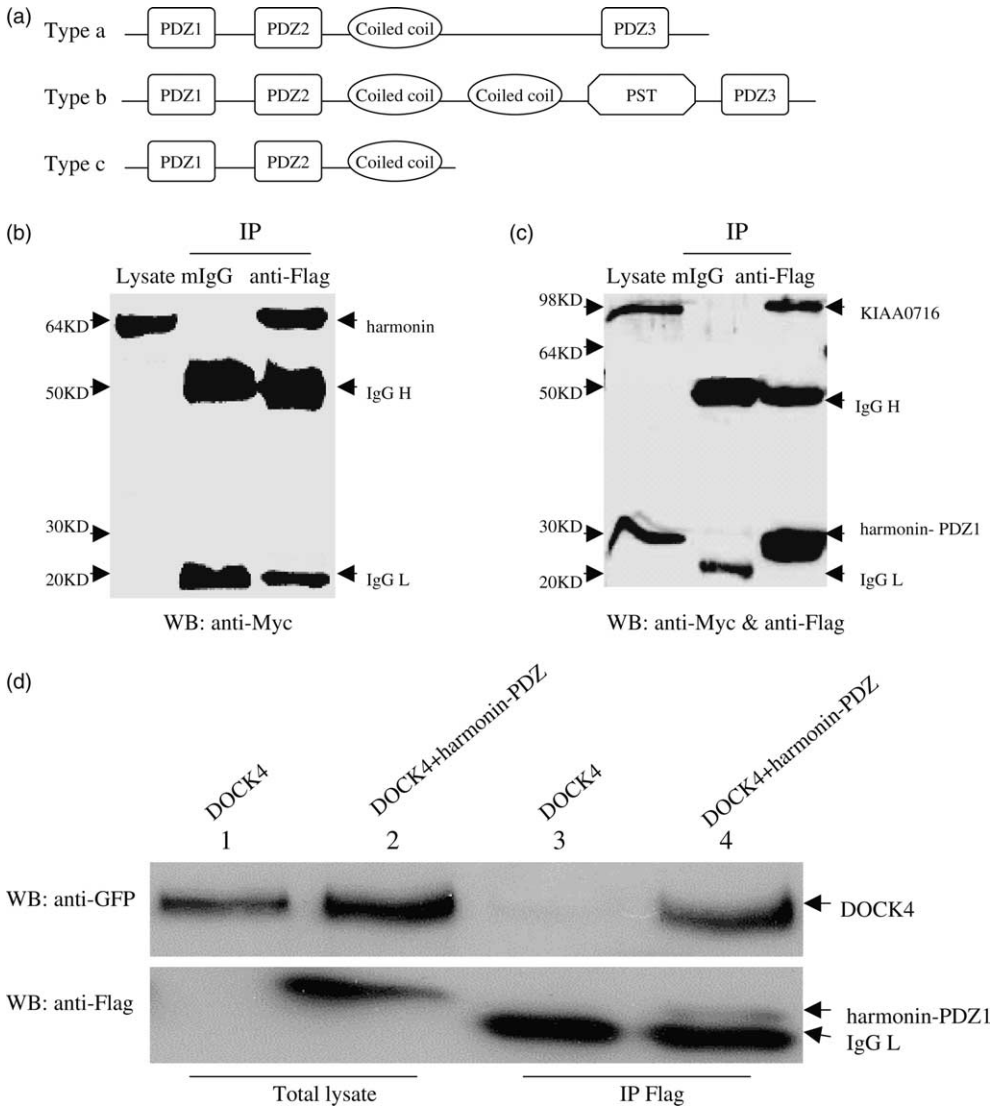

Figure 1. Co-immunoprecipitation studies confirming the interaction of harmonin with DOCK4. (a) A schematic diagram of the domain structures of the three major subclasses of harmonin isoforms. (b) HEK293T cells were co-transfected with pCMV-Flag-KIAA0716 (DOCK4) and pCMV-Myc-harmonin. After 24 h, the lysates were immunoprecipitated (IP) with anti-Flag monoclonal antibody or control mouse IgG. The presence of co-immunoprecipitated harmonin was assessed by Western blot (WB) analysis with anti-Myc antibody. (c) HEK293T cells were co-transfected with pRK-Myc-harmonin PDZ1 and pCMV-Flag-KIAA0716. The interaction was analyzed by subjecting the cell lysates to immunoprecipitation using anti-Flag monoclonal antibody and Western blotting using both anti-Myc and anti-Flag antibodies. (d) HEK293T cells were transfected with GFP-wild-type DOCK4 (lanes 1 and 3) or co-transfected with GFP-DOCK4 and pRK-Flag-harmonin-PDZ1 (lanes 2 and 4). Cell lysates were immunoprecipitated with anti-Flag antibody. The co-precipitated proteins were assessed by Western blot (WB) analysis using anti-GFP (top panel) or anti-Flag (bottom panel) monoclonal antibodies. Total cellular lysates from transfected cells were included in the blot to determine levels of expression of the transfected proteins as well as their endogenous expression.

20 independent recombinants revealed that seven clones corresponded to *KIAA0716*, characterized by the lack of the exon 49 while the remaining clones contained the exon 49. We observed an expected 400 bp PCR fragment using primers flanking the exon 49 on *DOCK4* wild-type cDNA, whereas a shorter product of 388 bp was observed with *KIAA0716* as template. When these primers were tested in a RT-PCR reaction using total RNA isolated from different human tissues (Invitrogen; multi-tissue RNA panel) (Figure 2(b)), the 400 bp band (lane 10) corresponding to wild-type DOCK4 is observed in all lanes (1–9, heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, respectively). However, the 388 bp band (lane 11) corresponding to KIAA0716 is only observed in total brain RNA (lane 2). In lanes 1 and 6, an intermediate band was observed. To investigate its origin, the PCR product was extracted, cloned and sequenced. The sequence analysis revealed that it is a result of trace DNA contamination in commercially bought RNA

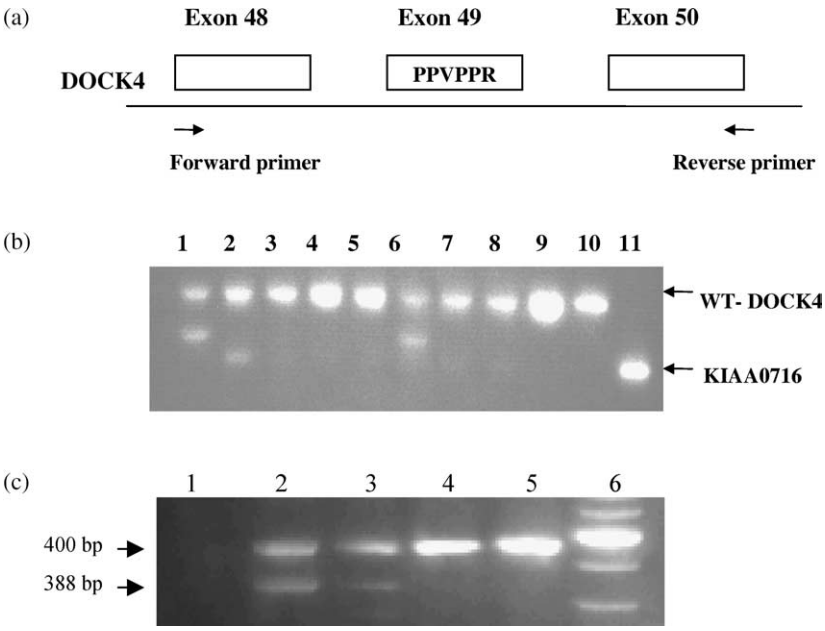

Figure 2. Expression analysis of DOCK4 by RT–PCR. (a) Schematic representation of the primers flanking the exon 49 of *DOCK4* wild-type cDNA. (b) RT–PCR with primers flanking exon 49 of *DOCK4* using RNA from multiple human tissues: lanes 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas; 9, spleen; 10, WT-*DOCK4* cDNA; 11, *KIAA0716* cDNA. An expected 400 bp PCR product corresponding to the WT-*DOCK4* sequence was observed in all lanes, whereas a shorter product of 388 bp was detected with *KIAA0716* as template (lane 11) and in total brain RNA (lane 2). (c) RT-PCR with primers flanking exon 49 of *DOCK4* using RNA from the brain in the absence (lane 1) or presence of reverse transcriptase in the reaction (lane 2); 3, eye; 4, heart; 5, kidney. A 388 bp product, characteristic of *KIAA0716* (DOCK4-EX49) was detected in the brain and eye.

leading to amplification of intron 50 and therefore is not a novel splice product. To investigate the expression of DOCK4 in the eye, RNA sample from human eye tissue was subjected to RT-PCR reaction along with RNA samples from brain, heart and kidney, using primers flanking exon 49 of the *DOCK4* wild-type cDNA. As shown in Figure 2(c), a 388 bp product, characteristic of *KIAA0716* (DOCK4-EX49) is only detected in the brain and eye (lanes 2 and 3) and absent in heart and kidney (lanes 4 and 5). Altogether these results therefore show that the DOCK4-EX49 isoform is selectively expressed in the brain and eye.

DOCK4-Exon49 is a strong Rac GEF

Members of the CDM family are unconventional, Rac GEF. These proteins bind to nucleotide-free Rac *via* the conserved DHR2/docker domain of 550 amino acids.[18] A steric hindrance model whereby the N-terminal SH3 domains of DOCK proteins exert an auto-inhibitory effect has been recently proposed[14] to explain the mechanism of Rac activation. To date, the role of the DOCK C-terminal serine/proline-rich region on the Rac GEF activity has not yet been studied. The splice variation between DOCK4 and DOCK4-Ex49 is present at the serine/proline-rich C terminus and therefore the DHR2 (amino acid residues 1134–1587) domains for both proteins are identical. To test whether DOCK4-Ex49 binds to nucleotide-free Rac, we performed a pull-down assay with Rac bound to glutathione *S*-transferase (GST-Rac) as bait. We also prepared a DHR2 deletion mutant by generating an in-frame 84 amino acid residue (1347–1430) deletion within the DHR2 sequence, resulting in a protein that is near full length in size and would act as a negative control in Rac binding and activation assays. HEK293T cells were transfected with Flag-tagged DOCK2, DOCK4, DOCK4 delDHR2 and DOCK4-Ex49. Cell lysates were used for a pull-down assay and subsequently analyzed by Western blot analysis using anti-Flag monoclonal antibody. As expected, DOCK4 wild-type and DOCK2 bind to nucleotide free Rac in this assay while DOCK4 with internal deletion in DHR2 domain (ΔDHR2-DOCK4) shows complete loss of binding capacity to nucleotide free Rac and there is a minimal GTP bound Rac in untransfected HEK293T cells. DOCK4-Ex49 binds to nucleotide free Rac as effectively as DOCK2 and DOCK4. Therefore, the splice variation in DOCK-Ex49 does not affect the binding ability of the protein to Rac *via* the DHR2 domain (Figure 3(a)).

We next determined the Rac activation by measuring the level of GTP-bound Rac by pull-down of the CRIB domain fusion (GST-PAK CRIB) that contains the Rac-GTP binding domain of PAK. The ratio of GTP-bound Rac to total cellular Rac was determined by Western blot analysis (Figure 3(b)). As expected, in positive control lanes containing samples transfected with GEFs DOCK2 and DOCK4, the level of GTP Rac increases while the deletion of DHR2 causes a significant decrease in the level of active Rac. In samples transfected with DOCK4-Ex49, there is an increased level of Rac activation over that of cells transfected with DOCK4 and DOCK2. These experiments were performed in triplicate and subsequently quantified (Figure 3(b) and (c)). Our results indicate that the DOCK4-Ex49 isoform is a potent Rac activator in comparison to the DOCK4 common isoform and closely related

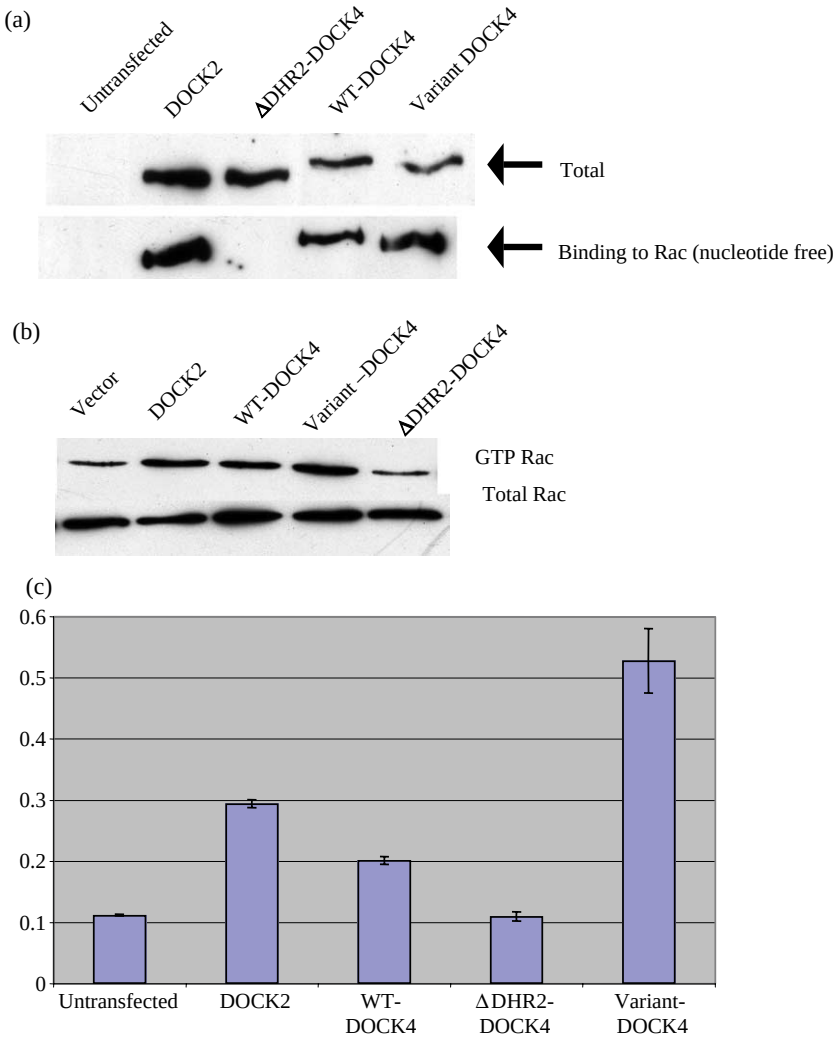

Figure 3. Rac-GEF activity of the variant-DOCK4. HEK293T cells were transfected with various constructs as indicated. (a) Rac binding. Cell lysates transfected with Flag-tagged clones were used for a pull-down assay with Rac-GST, followed by Western blot analysis with anti-Flag monoclonal antibody. The variant-DOCK4 was found to bind nucleotide-free Rac as effectively as DOCK2 and WT-DOCK4 while ΔDHR2-DOCK4 exhibited complete loss of binding to nucleotide free Rac. (b) Rac activation. Cell lysates were used for a pull-down of the CRIB domain fusion (GST-PAK CRIB). Total Rac and GTP bound levels were determined by Western blot analysis with anti-Rac antibody. DOCK2, WT-DOCK4 and the variant-DOCK4 activate Rac while the ΔDHR2-DOCK4 domain abrogates Rac activation. (c) Quantitative analysis of Rac activation by the variant-DOCK4, compared with DOCK2 and WT-DOCK4.

lymphocyte-specific DOCK2. It is possible that the C-terminal serine/proline-rich domain, alike the N-terminal SH3 domain of DOCKs influences the Rac GEF activity exerted by the DHR2 domain. However, it remains to be determined whether the present data will have an effect on the steric hindrance hypothesis of GEF activation. Of note, co-transfection of wild-type DOCK4 and harmonin full length into 293T cells does not affect the Rac-GEF activity of DOCK4. This finding would imply that binding of harmonin at the C terminus of DOCK4 does not lead to altered protein folding that may affect Rac activation property of DOCK4. It may also be an indication that DOCK4 works upstream of harmonin to mediate a branch of the Rho pathway to the regulation of the actin structure.

Generation of DOCK4 isoforms specific antibodies

Two peptide antibodies were generated: DOCK4-Ex49 is specific against a peptide (SAPEKAS-PARHT; amino acid residues 1755–1766), derived from the splicing of the exon49 of *DOCK4* and an antibody against a common sequence (RRTDPGPRPRPLPRKVSQL; amino acid residues 1948–1966) in the C-terminal tail of DOCK4 that would recognize both isoforms. The specificities of the antibodies were determined by Western blot analysis (Figure 4) using lysates from HEK293T cells (lane 1), and HEK293T cells transfected by either Flag-DOCK4-Ex49 (lane 2) or Flag- DOCK4 (lane 3). As a positive control for transfection, Western blot with anti-Flag antibody was performed to detect DOCK4-Ex49 (lane 2) and DOCK4 (lane 3). The C-terminal antibody common to both isoforms detects the over-expressed DOCKs (lanes 2 and 3). It is also able to detect the endogenous DOCK4 protein present in HEK293T cells (lane 1), whereas the isoform-specific antibody only recognizes the transfected DOCK4-Ex49 (lane 2) and does not cross-react with either endogenous DOCK4 (lane 1) or over-expressed DOCK4 (lane 3). These results confirm the specificity of the antibodies and indicate that they are a useful tool to investigate the expression of DOCK4 isoforms in inner ear.

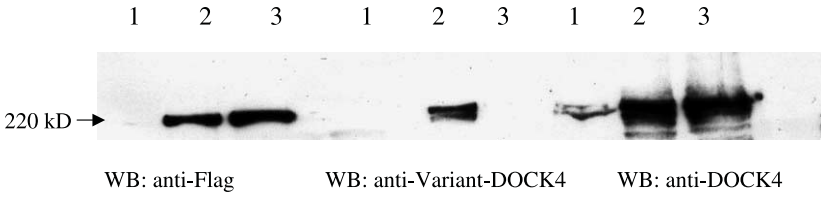

Figure 4. WT-DOCK4 and the variant DOCK4-Ex49 specific antibodies. The variant DOCK4-Ex49 specific antibody is generated against SAPEKASPARHT derived from the splicing of exon 49 of WT-DOCK4. The antibody that would recognize both isoforms was raised against a common sequence (RRTDPGPRPRPLPRKVSQL) in the C-terminal tail of DOCK4. The specificities of the antibodies were determined by Western blot analysis using lysates from HEK293T cells (lane 1), and HEK293T cells transfected by Flag-DOCK4-Ex49 (lane 2) or Flag-DOCK4 (lane 3). The C-terminal antibody common to both isoforms detects the over-expressed DOCKs (lanes 2 and 3), as well as the endogenous DOCK4 protein in HEK293T cells (lane 1), whereas, the isoform-specific antibody only recognizes the transfected DOCK4-Ex49 (lane 2).

DOCK4 is present in the hair bundles of the sensory cells (stereocilia) and is not mis-located in harmonin-deficient mice

To further characterize the expression of DOCK4-Ex49 in the inner ear, we studied its distribution in auditory sensory cells of $+/Ush1c^{dfcr\text{-}2j}$ single heterozygous controls and $Usher1c^{dfcr\text{-}2J}/Usher1c^{dfcr\text{-}2J}$ deaf circler mice. The *dfcr-2J* mutation is a 1 bp deletion at position 4 of exon C that causes a frameshift that changes the codons for 38 amino acid residues before ending at a stop codon at the beginning of exon D. It affects only isoform b transcripts of the *Usher1c* gene.[20] In the mouse, stereocilia sprout from the apical surface of vestibular and cochlear hair cells at E13 and E15, respectively.[21,22] During development, Dock4 protein was detected, using the isoform-specific antibody, in the utricular hair bundles as early as E14.5 (data not shown). The immunoreactivity maintained in the post-natal hair bundles. In a post-natal day 1 (P1) mouse, Dock4 labeled cochlear hair bundles of the sensory cells and the staining extended to the apical surface of the greater epithelial ridge (GER) (Figure 5(a)), whereas, in the vestibule, the Dock4 labeling was restricted to hair bundles (Figure 5(c)). Both in the cochlea and in the vestibule of mutant mice, the distribution of Dock4 was indistinguishable from that observed in $+/Ush1c^{dfcr\text{-}2j}$ single heterozygous controls (Figure 5(b) and (d)). There was no staining detected with the antibody against the C-terminal of DOCK4 that recognizes the common isoform. Previous immunohistolabeling analyses have shown the presence of harmonin in all the hair cells of the inner ear; the protein is distributed throughout the cell body and is localized in the stereocilia.[4,23] Together, those findings and our present data indicate that harmonin and DOCK4 have similar stereocilia localization in the inner ear. Our results also indicated that disruption in the *Ush1c* gene did not lead to altered expression or mis-localization of Dock4. Therefore, a functional harmonin is not required for the targeting and tethering of DOCK4 to the stereocilia that may be an indication that harmonin works downstream of DOCK4 to mediate a branch of the Rho pathway to the regulation of the actin structure. Previous studies suggest that there is a molecular pathway linking receptor molecules, such as integrins and/or Rho-type GTPases, downstream effector molecule diaphanous-1 (DIA1) into a signaling network that regulates the assembly or maintenance of the hair cell cytoskeleton.[24,25] Our current data imply that DOCK4, a Rho GEF, *via* its binding with harmonin that interacts with F-actin, may be involved in the actin cytoskeleton organization in stereocilia. Together, these results suggest a possible Rac-DOCK4-harmonin activated signaling pathway and that DOCK4 functions upstream of harmonin in regulating actin organization in the inner ear.

Discussion

Regulation of the dynamic behavior and assembly of the cytoskeletal filaments are important for providing the driving force for cell morphology. This phenomenon is achieved through a tight coupling between cell structure and signal transduction, a process that is regulated by a cohort of actin-binding proteins (ABPs), now known to be regulators of cellular dynamics and key components of signaling processes. At the core of stereocillia (SC), also known as hair bundles, is a parallel actin bundle (PAB) composed of actin filaments of uniform polarity.[26] PABs have been shown to contain different complements of actin-bundling proteins,[27] but little is known about what each contributes. However, there are many indications that each actin-bundling protein plays an important and specific role, as mutations that impair or eliminate different actin-bundling proteins have distinguishable effects on PAB organization.[28–30] The importance of SC morphogenesis becomes apparent in USH1. Mouse models of this disease show deafness and vestibular dysfunction with abnormal SC that are irregular in height and are splayed, suggesting a lack of lateral interactions.[31] How the individual gene products generate a common phenotype remains unclear, but it is likely that the SC defects are a result of disruption of molecular interaction amongst the Ush gene products and/or also of the actin cytoskeleton. Notably, early studies have shown that harmonin can bundle actin filaments, both *in vitro* and when overexpressed in fibroblasts.[23] The F-actin-bundling property of harmonin shown

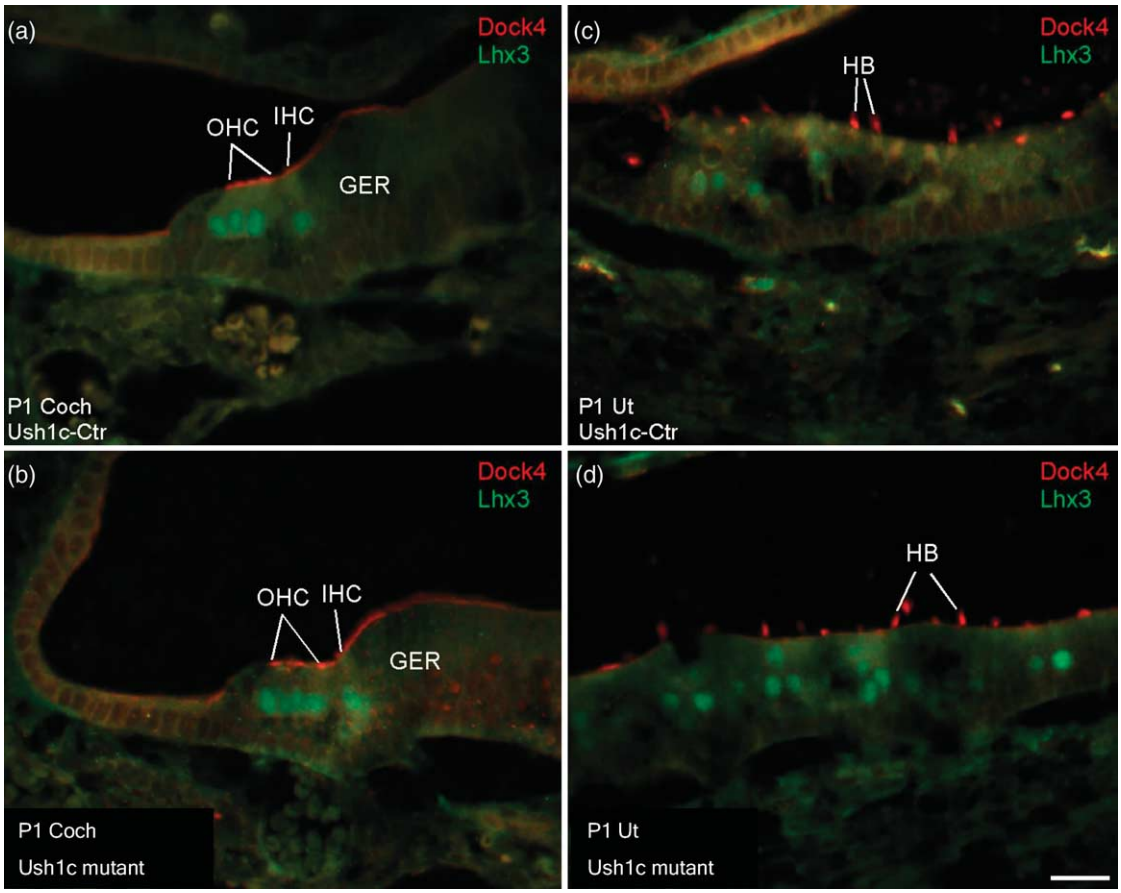

Figure 5. Immunohistochemistry of Dock4 in P1 mouse inner ear. (a) Dock4 labeled P1+/*Ush1c*$^{dfcr\text{-}2j}$ single heterozygous controls cochlear hair bundles, with Lhx3 labeled hair cell nuclei. The staining of Dock4 also extended to apical surface of the greater epithelial ridge (GER), next to the organ of Corti. (b) Similarly in P1 *Ush1c*$^{dfcr\text{-}2j}$/*Ush1c*$^{dfcr\text{-}2j}$ mouse cochlea, Dock4 labeled hair bundles and GER; (c) Dock4 labeled hair bundles (HB) of P1 control utricle; (d) Dock4 labeled HB in *Ush1c*$^{dfcr\text{-}2j}$/*Ush1c*$^{dfcr\text{-}2j}$ utricle, similar to control. OHC, outer hair cell; IHC, inner hair cell; Coch, cochlea; Ut, utricle. The scale bar represents 20 mm.

in vitro suggests that harmonin is implicated in the dynamics of the developing SC, by stabilizing the elongating actin filaments and contributing to the stiffness of the SC.[23]

To further understand the critical role of the scaffold protein harmonin in SC formation, we pursued a yeast two-hybrid screen with a brain cDNA library and identified DOCK4, an unconventional GEF, as harmonin interacting protein. DOCK4 is a member of the CDM gene family. The CDM proteins significantly differ at the C terminus and may provide specificity in cellular signaling. We propose that the divergent, proline-rich C terminus, contributes to the localization of the DOCK signaling complex to distinct sub-cellular destinations *via* binding to specific adaptor proteins. Here, we show that as a result of alternative splicing, an isoform of DOCK4 is present in the cochlear hair cells and the protein is localized to the SC. Alternative splicing can result in the formation of new epitopes or elimination of existing epitopes, or possibly both. These epitope or peptide motifs could lead to distinct protein–protein interactions resulting in novel sub-cellular destinations. As the DHR2 domain in the two isoforms is invariant, our results indicate that both isoforms are able to bind and activate Rac. Although quantitatively, the fold Rac activation observed for the DOCK4-Ex49 isoform is higher than for the common form of DOCK4. It is possible that the C terminus may also regulate the GEF activity analagous to that described for the N terminus. It is not clear whether the harmonin interaction is solely responsible for DOCK4-Ex49 localization to the stereocilia or there are other proteins that contribute to this distribution.

Key downstream targets for integrins and other cell-surface receptors including small GTPases of the Rho family such as RHO, RAC and CDC42 regulate many aspects of cytoskeleton dynamics in different cell types.[32] Although the function of these molecules in hair cells is unclear, the analysis of mutant mice suggests that one integrin, α8β1 and focal adhesion kinase (FAK), a key component of integrin-activated signaling pathways, are in a molecular pathway that regulates the assembly or maintenance of the stereocilia cytoskeleton.[24,25] Previous studies have shown that mutations in

known targets of Rho GTPases result in deafness further substantiating the role of small GTPases in the cochlea; mutations in the gene encoding diaphanous-1 (Dia1), a profilin ligand and target of Rho GTPase[33,34] lead to the autosomal dominant non-syndromic deafness DFNA1.[35] The DOCK4 related protein, DOCK180, is activated by integrin signaling leading to an interaction between its C-terminal proline-rich domain and the SH3 domain of the adaptor protein CrkII.[36] The SH2 domain of CrkII links this complex to the scaffold protein p130 CAS, which is phosphorylated by Src and FAK, triggering the cytoskeletal changes characteristic of Rac activation. Similarly, the proline-rich C-terminal domain of DOCK4 may play a similar role in CrkII binding and GTPase signaling.[10] It is possible that our observation linking harmonin to DOCK4 may constitute a branch of the integrin signaling pathway to the regulation of the actin cytoskeleton in the developing SC.

Our present data suggest an association between *DOCK4*, a member of the CDM gene family and the cytoskeleton, through its binding *via* its C terminus to a PDZ domain-containing protein, harmonin, that directly interacts with F-actin. Taken together, this suggests a possible Rac-DOCK4-harmonin activated signaling pathway in the inner ear.

Materials and Methods

Yeast two-hybrid screening

The yeast two-hybrid system Matchmaker II from Clontech (Palo Alto, California) was used according to the manufacturer's protocol. The bait encoding PDZ1-CC1 (amino acid position 6–448; BC016057) of the USH1C protein, obtained by PCR from a human brain library, was inserted in-frame into GAL4 DNA binding domain in the yeast expression vector pBridge. The resulting construct pBridge-Ush1C (PDZ1-CC1) was then used to transform the yeast strain AH109 by the lithium acetate method followed by selection on tryptophan-deficiency media. The positive transformants were subsequently transformed with a human brain cDNA library fused to the GAL4 transactivator domain in pACT2 vector. Yeast were cultured for five to ten days at 30 °C on a synthetic medium lacking tryptophan, leucine, and histidine. Grown yeast colonies were replica-plated on a medium lacking tryptophan, leucine, histidine and adenine. The clones that both grew on quadruple-deficient plates and were positive following the β-galactosidase assay were selected and their plasmids were isolated. GAL4 primers were used for PCR amplification of the positive clones and sequencing by the Perkin-Elmer ABI 373 DNA Sequencer (PE-Applied Biosystems, Foster City, CA) with Big Dye™ chemistry.

Mammalian expression constructs

N-terminal Myc or Flag-tagged pCMV expression vectors (Strategene, La Jolla, CA), Myc-or Flag-epitope-tagged pRK vectors and pEGFP-C2 vector (Clontech) were used to produce Ush1C and DOCK4 mammalian expression clones. The *USH1C* gene constructs were obtained by sub-cloning the PCR fragments generated from a human kidney library with (sense/anti-sense primers) for full length 5′-GCAACGACG CAGCTGGACCT-3′/5′-CGGTGAATTTGGTTTCCC-3′ (BC016057); 5′-CATGGACCGAAAAGTGGCCCGAG-3′/ 5′-ATCCACATACTGCCAAGTGA-3′ for the PDZ1 domain. The C-terminal domain (amino acid positions 1150–1966; AB018259) of human DOCK4 was produced by PCR from KIAA0716 (clone kindly provided by Dr Takahiro Nagase of the Kazusa DNA Research Institute) with 5′-TGTGGATCCATGGCGGGAAAGTGGCGTT-3′/ 5′TAACTGAGAGACCTTGCGGG-3′. The purified products were digested with appropriate enzymes and ligated into the expression vectors.

Cell culture and transfection

All culture media were from Life Technologies-Gibco (Rockville, MD, USA). The human embryonic kidney (HEK) 293T cell line was maintained in high glucose Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum, 100 μg/ml penicillin G and 100 μg/ml streptomycin. For fluorescent microscopy, cells were seeded on eight-well plastic chamber slides. For Western blot and co-immunoprecipitation experiments, cells were seeded on 10 cm dishes. Cells were transfected the following day by the standard calcium phosphate precipitation. At 24–48 h after transfection, cells were used for immunocytochemistry and Western blot analysis.

Immunoprecipitation and Western blot analysis

Transfected HEK293T cells were solubilized in 1.5 ml of Triton X-100 based lysis buffer containing 20 mM Tris–HCl (pH 7.4), 1% (v/v) Triton X-100, 1 mM EDTA, 150 mM NaCl, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 30 mM sodium fluoride and 2 mM sodium orthovanadate for 40 min at 4 °C. The nuclear and cellular debris were cleared by centrifugation at 20,000*g* for 20 min at 4 °C. The lysates were incubated overnight at 4 °C with 0.4 μg of antibody or control IgG. The immune complexes were precipitated with Dynal magnetic Protein G (Dynal, Lake Success, NY, USA), washed four times with 0.6 M NaCl lysis buffer, and then eluted by boiling for 5 min in SDS sample loading buffer. The eluted proteins were separated on SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The immunoprecipitated proteins were detected by Western blot analysis using enhanced chemiluminescence Western blotting detection reagents (Amersham, Piscataway, NJ, USA). The total lysates were also resolved on SDS-PAGE and detected by Western analysis.

Reverse transcription polymerase chain reaction (RT-PCR)

To identify the expression of DOCK4 isoform in various human tissues, the following 5′-GAGACCATGCAGTGC CATCTATC-3′/5′-AGGTTTCCGAGCTGCTGCACC-3′ primers were used on Clontech (BD Sciences) multi-tissue panels I and II according to the manufacturer's instructions. Total RNA was purified from adult C57BL/6 mouse eye and inner ear tissues with TRIzol reagent (Invitrogen). Mouse cDNA was prepared from total RNA with the SuperScript First Strand cDNA synthesis System for RT-PCR (Invitrogen). One-tenth volumes of the first strand synthesis reactions were amplified by PCR.

Rac binding and Rac activation assays

Clarified lysates from HEK293T cells were prepared 36 h after transfection with Fugene (Roche). A total of 6 μg of DOCK plasmids were used to transfect 10 cm plates. Cells were lysed in 1% Triton X-100, 500 mM NaCl, 10 mM $MgCl_2$ and with complete protease inhibitor (Roche). Nucleotide-free Rac was prepared as a GST fusion protein, as described.[18] Lysates were incubated with 5 μg GST-Rac, nucleotide-free, at 4 °C for 4 h and samples were washed four times in Triton X-100 lysis buffer. Bound Flag-tagged DOCK protein was analyzed by Western blot analysis. The intracellular Rac-GTP level was assessed by precipitation with GST-CRIB beads and immunoblotting for Rac. Rac-GTP levels were normalized against the expression level of Rac and DOCK proteins and the quantitation was determined from four independent experiments.

Antibodies

Anti-Flag monoclonal antibody was obtained from Sigma (St. Louis, MO, USA). Anti-Myc monoclonal antibody was from clontech (Palo Alto, CA, USA), and anti-Lhx3 was obtained from DSHB (University of Iowa). The antibody to DOCK4 was generated against the peptide RRTDPGPRPRPLPRKVSQL (1948–1966) corresponding to the C terminus amino acid sequence (Invitrogen). The isoform (DOCK4-Ex49)-specific antibody was to the peptide sequence SAPEKASPARHT (1755–1766), corresponding to the amino acid sequence created by splicing exon 49 of *DOCK4* (Invitrogen).

Immunohistochemistry

Post-natal day 1+/$Ush1c^{dfcr\text{-}2j}$ single heterozygous controls and $Ush1c^{dfcr\text{-}2j}/Ush1c^{dfcr\text{-}2j}$ homozygous mice were obtained from Jackson Laboratory. Frozen sections of the mouse inner ear tissues were prepared for immunolabeling. The slides were dried for 15 min at 37 °C, and re-hydrated in 1XPBS for 5 min. The sections were then subjected to an antigen unmasking treatment using the Antigen Unmasking Solution (Vector Laboratories) according to the manufacturer's protocol. The blocking, primary antibody and secondary primary antibody incubation were performed according to the standard protocol. The secondary antibodies were anti-rabbit alexa 594 and/or anti-mouse alexa 488 (Molecular probes). Data visualization and acquisition were performed using a regular fluorescent microscope (Zeiss, Axioscope 2).

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